British Journal of Pharmacology

4

January 1976

filing

12/5

NOT TO BE LENT OUT

Edited for the British Pharmacological Society by

A.W. Cuthbert *(Chairman)* H.M. Adam *(Secretary)*
Margaret Day *(Press Editor)*
J.D. Stephenson *(Assistant Press Editor)*

A.M. Barrett
W.G. Bisset
T.B. Bolton
P.B. Bradley
D.A. Brown
B.A. Callingham
T.J. Crow
F. de Matteis
C.T. Dollery
V. Eisen
E.W. Gill
A.F. Green
D.F. Hawkins
Margarethe Holzbauer
D.B. Hope
J.S. Kelly
G.M. Lees
G.P. Lewis
I.H.M. Main
E.K. Matthews
J.F. Mitchell
P.A. Nasmyth
M.W. Parkes
M. Sandler
A.P. Silverman
M.A. Simmonds
F.M. Sullivan
A. Ungar
R.A. Webster

P25830

Papers will be considered for publication on all aspects of drug action, including chemotherapy.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh EH8 9JZ. Authors should consult the instruction inside the back cover.

The British Journal of Pharmacology is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633. ISSN 0007-1188.

Annual subscription £36.00 ($90 USA and Canada). (Orders must be accompanied by remittance. Prices applicable only to orders received before 31st December 1976.)

Cheques should be made payable to Macmillan Journals Limited: Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

Enquiries concerning advertising space and rates should be addressed to: Advertisement Department, Macmillan Journals Limited, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633.

Copyright © 1976 by the *British Journal of Pharmacology.* All rights of reproduction are reserved in respect of all papers, articles, illustrations, etc., published in this journal in all countries of the world.

Second class postage paid at New York, N.Y.
US Mailing Agent, Air and Sea Freight Inc., 527 Madison Avenue, New York, NY 10022.

DIAGNOSTIC PHARMACEUTICAL DEVELOPMENT

From £5150
To lead Animal Physiology Section

This is a career development opportunity in a high technology fast growth industry. We want a professional Scientist with innovative flair and practical experience in Physiology to take charge of animal work carried out on small mammals The section to be controlled is concerned with developing new medical products, preparing anti-sera for use in clinical diagnostic systems and some quality control testing of existing materials. There are currently eight people in the section, including professionally qualified staff.

You will need creativity as well as organising ability to contribute effectively to the development of new products and to work within a multi-discipline development section.

At least a good class Honours Degree in an appropriate subject plus practical experience in animal experimentation are essential prerequisites. Acquaintance with Nuclear Medicine or the use of Radioactive materials will be useful, as will experience of supervising a team, or working in close collaboration with others

Conditions of employment are excellent and include a contributory Superannuation Scheme, Sickness Benefit Scheme, Generous Holidays, Staff Canteen and a Sports and Social Club

Applications should be sent to S. F Rush, Recruitment Section, The Radiochemical Centre, Amersham, Bucks, or telephone Little Chalfont 4621 (reverse charge) for more information and an application form.

Amersham is situated in a picturesque green belt area of Buckinghamshire, and is surrounded by many places of historic interest It has easy access to London by road and rail, being close to the M1, M4 and M40 motorways Both housing and educational facilities reach a high standard, and the wide range of sporting activities available combine to make this a most desirable area in which to live

The Radiochemical Centre

Amersham Bucks

1967 1970 1974

Pitman Medical

ADVANCED MEDICINE
Topics in Therapeutics—1

Edited by A.M. BRECKENRIDGE, *MD, MSc, FRCP*

The first volume in a new series of Symposia held by the Royal College of Physicians of London, being an extension of, and complementary to, the well-established Advanced Medicine symposia. The new series concentrates entirely on therapeutics, while the established series will continue to cover the wide range of the whole of modern medicine.

Contents: Problems of Poisoning and Drug Overdosage—New Problems Presented by Old Drugs—Medical Education and the Drug Explosion—Problems of Changing Therapeutic Practice—Problems Presented by Psychotropic Agents.

270 pages Illustrated Paperback £6.50 net

FITC-DEXTRANS
for permeability and microcirculation studies in animals

Well characterized tracer substances

- fluorescein label for easy detection
- ultra-micro determination in nanolitre sample volumes
- narrow molecular weight distribution
- five fractions between 3,000 and 150,000 daltons

FITC-Dextrans and other products for biological research are described in the booklet "Biomedical Research. Product guide", free on request.

Pharmacia (Great Britain) Ltd.
Paramount House,
75 Uxbridge Road,
London W5 5SS

Pharmacia
Fine Chemicals

British Journal of Pharmacology

VOLUME 56 NUMBER 1 JANUARY 1976

3 SCHILD, H.O. Dale and the development of pharmacology

9 HILL, R.G., SIMMONDS, M.A. & STRAUGHAN, D.W. Antagonism of γ-aminobutyric acid and glycine by convulsants in the cuneate nucleus of cat

21 GANGULY, D.K. Antioxotremorine action of propranolol

25 BOSIN, T.R., HIXSON, E. JANE & MAICKEL, R.P. Pressor effects of tryptamine analogues

9 OSBORNE, N.N. & PENTREATH, V.W. Effects of 5,7-dihydroxytryptamine on an identified 5-hydroxytryptamine-containing neurone in the central nervous system of the snail *Helix pomatia*

9 GRYGLEWSKI, R.J. & KORBUT, R. Bioassay of histamine in the presence of prostaglandins

43 ADAMS, M.D., CHAIT, L.D. & EARNHARDT, J.T. Tolerance to the cardiovascular effects of Δ^9-tetrahydrocannabinol in the rat

49 GILBERT, J.C. & WYLLIE, M.G. Effects of anticonvulsant and convulsant drugs on the ATPase activities of synaptosomes and their components

59 JOHNSON, A.M., LOEW, D.M. & VIGOURET, J.M. Stimulant properties of bromocriptine on central dopamine receptors in comparison to apomorphine, (+)-amphetamine and L-DOPA

69 BUCKINGHAM, R.E., HAMILTON, T.C. & MOORE, R.A. Prolonged effects of *p*-chlorophenylalanine on the blood pressure of conscious normotensive and DOCA/saline hypertensive rats

77 MILSON, J.A. & PYCOCK, C.J. Effects of drugs acting on cerebral 5-hydroxytryptamine mechanisms on dopamine-dependent turning behaviour in mice

87 JOHNSON, F.N. The effect of lithium chloride on one-trial passive avoidance learning in rats

93 BARBER, H.E., CALVEY, T.N., MUIR, K.T. & TAYLOR, K. The effect of edrophonium on erythrocyte acetylcholinesterase and neuromuscular function in the rat

101 STONE, T.W. Responses of neurones in the cerebral cortex and caudate nucleus to amantadine, amphetamine and dopamine

111 FELTZ, ANNE & LARGE, W.A. Effect of atropine on the decay of miniature end-plate currents at the frog neuromuscular junction

115 DANILOV, A.F., IVANOV, YU.J. & MICHELSON, M.J. The influence of motor nerve tetanization on the effect of organophosphorus cholinesterase inhibitors on neuromuscular transmission

SHORT COMMUNICATIONS

125 BAKHLE, Y.S. & YOUDIM, M.B.H. Metabolism of phenylethylamine in rat isolated perfused lung: evidence for monoamine oxidase 'type B' in lung

128 BROWN, D.A. & KWIATKOWSKI, D. A note on the effect of dithiothreitol (DTT) on the depolarization of isolated sympathetic ganglia by carbachol and bromo-acetylcholine

Br. J. Pharmac. (1976), **56**, 3–7

DALE AND THE DEVELOPMENT OF PHARMACOLOGY

Lecture given at Sir Henry Dale Centennial Symposium, Cambridge, 17–19 September 1975

H.O. SCHILD
Department of Pharmacology, University College, London

The title of my lecture, 'Dale and the development of pharmacology', seems entirely appropriate in view of Dale's fundamental contributions to the subject; but it also contains a paradoxical element since Dale did not really consider himself a pharmacologist. He considered himself a physiologist, as he was by aining and inclination. He trained in physiology with angley and Gaskell in Cambridge, and after ompleting his medical course at Barts continued orking as a physiologist with Starling as Sharpey holar. When at the age of 29 he became a pharmacologist by joining the Wellcome laboratories, he did not seem entirely happy at the transition, as is apparent from his writings in which he refers to the ergot 'morass' which he had entered, although he xtracted some marvellous nuggets from it.

Dale was atypical in his attitude of cautious reserve owards the drug receptor idea which has become so ominant a feature of pharmacology. He did not plain in terms of receptors the adrenaline reversal by got and although he knew of Langley's postulated eceptive substances' he tended to emphasize our total gnorance of their nature. Interestingly though, in his 919 Croonian Lecture he argued that the one eceptive substance of which we knew something (because it could be studied apart from the cell) was the anaphylactic antibody, suggesting that its reaction with a specific antigen might provide an opportunity of studying drug-receptor interactions in a 'test tube'.

On a deeper level, Dale at times seemed almost surprised at the long range pharmacological-medical implications of his own discoveries. On one occasion, when discussing the early clinical uses of ganglion blocking drugs in hypertension he expressed astonishment that a symptomatic drug could be so effective in a chronic disease, probably reflecting the philosophy of Ehrlich who distinguished sharply between symptomatic and causal drugs. Yet, we know now from statistical evidence that treating the symptom hypertension, prolongs life.

It can be said of Dale, as indeed of Ehrlich, that he was one of the great originators of pharmacology rather than a typical pharmacologist. Such people, by heir very power and originality, cannot readily be ccommodated within the framework of a restricted subject, though Dale's work is bound to exert a deep and lasting influence on the development and future of pharmacology.

Dale's laboratory

Dale's great discoveries were made with a few selected coworkers and assistants in the atmosphere of the typical old fashioned physiological laboratory, having the smoked drum as its mainstay. I would like to recall the impression he made on a young pharmacologist of little experience entering his laboratory for the first time. When I joined Dale in 1932 I had been working for a short while with Straub, a German pharmacologist concerned at that time with gastro-intestinal pharmacology. Entering Dale's laboratory from Straub's, one had the impression of coming in from the periphery and entering the centre. Every problem tackled in Dale's laboratory was central. He himself had recently completed an investigation on muscarinic transmission by acetylcholine; Feldberg and Gaddum were soon to start their study of nicotine transmission by acetylcholine in the superior cervical ganglion. I was studying adrenaline. von Euler and Gaddum had recently discovered substance P and we continued this work with Gaddum. Marks was studying the mode of action of insulin. These were all central problems at the time and have remained so after all these years.

Dale's laboratory was magnificently organized under the eagle eye of Collison whom one had to inform the evening before if one wanted to do an experiment. Next morning the experiment was perfectly set out by one of his highly trained technicians: but it wasn't advisable to change one's mind then and suggest a different experiment. Dale was the director of the entire National Institute for Medical Research, but he frequently visited, and sometimes experimented, in his own laboratory F4. The laboratory was permeated by his ideas; his conversation was informative and lucid as it remained to the end of his life. He was a keen experimenter himself; I can remember on one occasion fumbling with a scalpel to start a dissection in an anaesthetized cat and Dale practically taking the instrument out of my hand and finishing the dissection himself to show me how it ought to be done.

His scientific approach is perhaps best illustrated by one of Dr Feldberg's characteristic stories told at a student dinner. He was comparing the differing approaches of three eminent scientists, Langley, Barcroft and Dale, all of whom he knew well. If Langley had done five experiments, he said, and the sixth gave a divergent result he would do another ten. Barcroft in the same situation would concentrate on the last as being the most interesting. But Dale's style was different. He believed in working like an astronomer, thinking out and planning the work in greatest detail and finally settling the issue with a few carefully chosen experiments.

I had an accidental glimpse of Dale's approach on an occasion when I visited the National Institute on a trip to London. I found Dale and Feldberg engaged in a fundamental experiment in which they were trying to demonstrate that sweat gland secretion, although anatomically innervated by the sympathetic, was cholinergic. They perfused the footpad of an anaesthetized cat with eserinized Ringer and were going to stimulate the sympathetic chain and use the dorsal muscle of the leech as a test for acetylcholine release. They duly stimulated the sympathetic but could find no evidence of acetylcholine release. On that day Dale was not in the best of tempers and he left the laboratory abruptly. Next day I visited the laboratory again and found out that the experimenters had decided to change their approach in a small but vital respect by doubling the concentration of potassium in the perfusate. This time there was a large contraction of the leech after stimulation of the sympathetic chain: acetylcholine had been released. Dale was happy and jovial and said laughingly 'and if they don't believe it, let *them* repeat the experiment'. The experiment was of course repeated by the authors themselves, indeed it is recorded that it was repeated four times with identical results, but Dale's facetious remark shows how much store he set by the outcome of even a single experiment, if it was well thought out, and carefully executed.

Contributions to pharmacology

Dale has made many contributions to pharmacology and I shall select only a few that have particularly influenced future developments.

Adrenaline reversal by ergot

This was one of Dale's earliest discoveries. It is interesting to read in his semi-autobiographical *Adventures in Physiology* how at the age of 29, Dale found himself without a permanent academic post and how Henry Wellcome offered him a post in his establishment adding that he hoped he would do work permanent scientific value there.

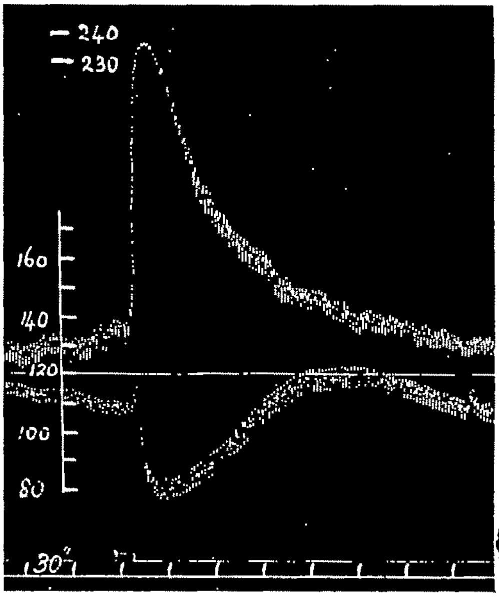


Figure 1 Pithed cat. Carotid blood pressure Upper curve shows the effect of 0.025 mg of adrenine before, lower curve that of 0.1 mg adrenine after 10 mg of ergotoxine. (From Dale, H H. (1913). On the action of ergotoxine: with special reference to the existence of sympathetic vasodilators. *J. Physiol. Lond.*, **46**, 291–300, reproduced by permission of *The Journal of Physiology*).

His first assignment was an investigation of th pharmacology of ergot of which he soon tired since a he could discover was that all ergot extracts prepared by his friend Barger produced a rise of blood pressure in the spinal cat. Then, by one of his 'greatest strokes of good fortune' he had the opportunity of committing a 'howler' when he was sent a sample of dried suprarenal powder from the factory with a request to test it for adrenaline. He tested it on a cat that had received ergot all day and could get no rise of blood pressure with it, only a fall. So he pronounced it inactive; this was the howler. Next week by another fortunate coincidence—but he adds slyly, perhap they also wanted to test the competence of their newly-found pharmacologist—he was sent another suprarenal extract and the same sequence of events repeated itself. Dale now became suspicious and investigated the interactions of adrenaline and ergot in a remarkable series of experiments the main outcome of which is summarized in Figure 1 which shows the phenomenon of adrenaline (adrenine) reversal by ergot. Adrenaline alone produced a rise of bloo pressure in the spinal cat but after ergot it produced fall. Dale concluded that ergot had paralyzed the myoneural junctions of the sympathetic concerned with motor function, leaving those concerned with inhibition unaffected. In the same paper Dale showed that the cardioaccelerator effect of adrenaline was not, or was much less, antagonized by ergot. The paper thus goes a long way, though by no means the whole way, towards establishing what were later called by Ahlquist the alpha and beta actions of adrenaline. Perhaps the main difference in Dale's approach was that he did not analyze the problem in terms of receptors.

Sympathomimetic amines

The term *sympathomimetic* was introduced by Barger and Dale to denote a range of compounds which simulate the effects of sympathetic nerves with arying intensity and varying precision'. Their study f over 50 sympathomimetic amines was one of the ly comprehensive investigations of the relation ween chemical constitution and pharmacological ction, a subject which had always interested Dale. heir main conclusions were that the more substances resembled adrenaline in structure the more active they became, and that all sympathomimetic compounds were primary or secondary amines whilst tertiary and quaternary bases had nicotine-like actions.

Retrospectively one of the most interesting aspects of their paper, which was published in 1910, is its discussion of noradrenaline. Dale investigated noradrenaline which was then already obtainable from the Hoechst company under the name *arterenol* and found that it had greater stimulant and less inhibitory activity than adrenaline and he points out in the discussion that the actions of noradrenaline correspond more closely to sympathetic stimulation than those of adrenaline. He rejects Elliott's earlier suggestion that the similarity between the effects of adrenaline and sympathetic nerves may be due to the latter liberating adrenaline at nerve endings because of the lack of parellelism between adrenaline and sympathetic stimulation; but he fails to conclude that noradrenaline might occur as such in the body and be a better candidate than adrenaline for Elliott's postulated sympathetic transmitter substance. Dale himself marvelled later at his failure to 'jump to the truth'.

In this connection I can recall when working with Dale in 1933 and finding a consistent discrepancy between physiological and colorimetric estimates of adrenaline content of suprarenal medulla, that it did not seem to occur to either of us that the discrepancy might be due to the adrenal gland containing noradrenaline as well as adrenaline. It remained for the later work by Holtz and von Euler to establish clearly the occurrence of noradrenaline in the suprarenal medulla as well as in postganglionic sympathetic nerves.

Histamine

Dale was deeply interested in histamine, which he discovered with Barger in ergot extracts and whose pharmacological actions he described almost completely except its action on acid gastric secretion which was described by Popielski.

He was particularly interested in the capillary dilator effect of histamine, which he used as a tool to analyze capillary function under normal and pathological conditions. He considered histamine an important ingredient of 'autopharmacology' and introduced the notion of a 'locally acting chemical stimulant' to distinguish between true hormones in the sense of Bayliss and Starling and substances like histamine whose effects were restricted to the immediate neighbourhood of their liberation.

Dale considered the possibility that the release of histamine or a histamine-like substance might contribute to the physiological regulation of capillary blood flow, reasoning that the liberation of such a principle, as the result of metabolic activity, would provide a perfect fine adjustment of the capillary circulation to the needs of the tissues. But he remained reluctant to accept this 'teleological' argument as evidence and at no point does he in fact suggest that histamine is a physiological capillary regulator.

Dale was similarly cautious in accepting histamine as a cause of traumatic shock, although he had shown with Laidlaw that the injection of large doses of histamine in cats produced a condition of irreversible circulatory collapse due to fall in blood volume caused by a loss of tone of the capillaries, accompanied by an excessive permeability of blood vessels allowing the escape of plasma.

This cautious attitude was due partly to lack of evidence that histamine was a normal constituent of tissues, but even at a later stage, after his own laboratory had shown that histamine could be extracted from tissues, he would go no further than to admit that it might be involved as a capillary dilator in reactions of superficial injury such as Lewis's triple response. Dale's standards of proof were rigorous. Even histamine release in anaphylaxis he would only accept after Feldberg and Dragstedt had produced unequivocal direct evidence for it.

Although Dale failed to establish a physiological role for histamine his papers on histamine are amongst his most instructive, as examples of his scientific style, powers of physiological analysis and range of techniques. They also illustrate the penetrating quality of his discussion and his care in drawing conclusions from experiments.

Theory of neurohumoral transmission

The neurohumoral theory which Loewi and Dale created produced a fundamental change of outlook in which Dale's studies of acetylcholine have played a key role. Acetylcholine was synthesized in the 1890's and in 1906 Hunt and Taveau discovered its strong blood pressure lowering effect. They already considered the possibility that it might be a natural body constituent but this was not proved until much later, when Dale and Dudley extracted it from the spleen. Dale discovered acetylcholine, as he had discovered histamine, in ergot and in 1914 carried out an important investigation of its pharmacological properties. He noted a striking parallelism between the effects of acetylcholine and parasympathetic nerve stimulation and found that the effects of small doses of acetylcholine were readily antagonized by atropine but that larger doses given after atropine produced a nicotine-like stimulation of sympathetic ganglia and the adrenal medulla. These observations induced him to subdivide the actions of acetylcholine into 'muscarine' and 'nicotine' actions with far-reaching consequences for the future.

At that time Dale had not yet formed a clear notion of neurohumoral transmission. In a retrospective lecture he characterized the position around 1914 as follows: 'Two substances were known with actions suggestively reproducing those of the two main branches of the autonomic nervous system: both were very unstable in the body and their actions were consequently of a fleeting character and one of them was already known to occur as a natural hormone. These properties would fit them to act as mediators of autonomic impulses to effector cells if there were any acceptable evidence of their liberation at nerve endings. The actors were named and the parts allotted. A preliminary hint of a plot had indeed been given ten years earlier but had been completely forgotten (Elliott's hint that adrenaline might be the sympathetic transmitter), but only direct and unequivocal evidence could ring up the curtain and this was not to come until 1921'.

In 1921 Otto Loewi began his series of classical experiments on the frog heart which established the concept of neurohumoral transmission. The subsequent work by Dale and his associates was complementary to that of Loewi. It is almost impossible to disentangle the contributions of Loewi and Dale to the humoral theory, so closely are they interwoven. I believe that both are equally responsible. Dale would not have produced the theory without Loewi's brilliant experiments, and Loewi could not have produced it without Dale's deep physiological insight. Whereas Loewi had generated a brilliant general idea, Dale gave it life and substance by applying it to autonomic and somatic nerves and building up an integrated system of neurohumoral transmission in which the muscarinic and nicotinic actions of acetylcholine played a basic role.

The main discoveries of Dale's group, made in rapid succession, can be summarized as follows. Acetylcholine is the chemical transmitter of vagus effects to the stomach (Dale & Feldberg, 1934a). Acetylcholine mediates transmission from sympathetic postganglionic nerves to sweat glands (Dale & Feldberg, 1934b). (In this context Dale suggested the words 'cholinergic' and 'adrenergic' to provide a functional classification of nerve endings.) Acetylcholine transmits the stimulus of splanchnic nerves to the adrenal medulla (Feldberg, Minz & Tsutzimura, 1933). Acetylcholine transmits the preganglionic stimulus in the superior ganglion (Feldberg & Gaddum, 1934). Acetylcholine is the transmitter at the motor endplate of striated muscle (Dale, Feldberg & Vogt, 1936; Brown, Dale & Feldberg, 1936). Although the idea that the short-latency nicotinic transmissions at ganglia and the motor endplate are transmitted by acetylcholine was resisted at first by electrophysiologists, the neurohumoral theory became widely accepted by the end of the decade.

The work of Loewi and Dale led to a transformation of physiological and pharmacological thinking. The idea which had persisted since Claude Berna that curare blocked motor nerve endings, and t related ideas that adrenaline and pilocarpine stimulated sympathetic and parasympathetic nerv endings and that atropine blocked the latter had to be abandoned. A new pharmacology based on humoral transmission was developed with important consequences for clinical medicine. It now became possible to evolve by rational processes drugs capable of modifying neurohumoral transmission such as the anticholinesterases in myasthenia gravis, tubocurarine in anaesthesia and adrenergic neurone blocking drugs in hypertension. It is of historical interest that Burn & Dale (1915) showed that tetraethylammonium antagonized the ganglion stimulant effect of tetramethylammonium. Some 30 years later tetraethylammonium was used clinically as the first effective ganglion blocker for the treatment of hypertension.

Anaphylaxis

Dale's contribution to anaphylaxis was brief and incisive. Once again his initial discovery was made by accident, 'one of my most fortunate accidents' as he called it. Whilst experimenting with guinea-pig isolated uterus he came across a specimen which responded with a maximal contraction to a small dose of horse serum. On enquiry he learned that the guinea-pig was sensitized to horse serum having been used previously for antitoxin experiments. The finding that an isolated organ taken from a sensitized animal would respond to the specific antigen in the complete absence of serum or blood was the beginning of the cellular theory of anaphylaxis. Soon afterwards Dale found out that Schultz in Washington had independently made similar observations and he considered giving up the work. Fortunately he did not do so because although the reaction itself is rightly credited with both their names (it is generally referred to as the Schultz-Dale reaction in the U.S.A. and as the Dale-Schultz reaction in Britain), Dale's analysis of the underlying mechanism carried the day.

His analysis had the quality of diagrammatic simplicity. He demonstrated that the reaction was highly specific and that it depended on the antibody becoming firmly attached to the cell. He clearly distinguished between desensitization and immunity, defining desensitization as the failure of the sensitized tissue to respond repeatedly to the same antigen whilst immunity, he considered, was due to excess free antibody in the serum capturing the antigen and preventing its access to the sensitized tissue. Immunology has greatly altered since Dale's time and many of his detailed immunological concepts are no longer tenable, but his general conception of cellular anaphylaxis has stood the test of time largely unchanged.

Biological standardization

Dale said of biological standardization that 'for many years it made a claim on time and thought which I would have willingly given to more fundamental but not, I think, more important research activities'. Their importance lay in three directions. First, it was largely through Dale's insistence on the comparative nature of biological standardization and the necessity for stable standards that reliable samples of insulin, posterior pituitary and digitalis became available to the medical profession. Second, Dale formulated the principles of biological standardization in so lucid a manner that to this day they have formed the basis of teaching the subject. Third, and perhaps most important, was his indirect influence in developing the field of biological assay. He somehow managed to congregate around him or influence a group of mathematically inclined pharmacologists such as Treven, Gaddum and others who established the statistical principles of biological assay. Bioassay has become the basis of all pharmacological measurement in the widest sense and it is largely through the study of bioassay that statistical measuring techniques have entered pharmacology and medicine. Much of the present emphasis on the quantitative assessment of drug action can be traced back to this small group of fiercely independent workers who were yet all somehow associated with Dale.

Dale's impact

Dale's most outstanding contribution to pharmacology was undoubtedly neurohumoral transmission because of its general significance, but he has made many special contributions, amongst them discoveries of great importance, only a few of which I have been able to mention. For example he was the first to describe the stimulant action of posterior pituitary extract on the uterus.

It is astonishing how much of Dale's work still stands today. This is due in part, no doubt, to the careful way in which he hedges around his statements—he was known in his youth to rewrite his papers many times—but more basically it is due to the inherent quality of his achievement, its solidity and realism. His ideas have proved eminently fruitful as the present meeting testifies. It is astonishing how Dale's notions of neurohumoral transmission which were originally conceived in terms of the peripheral nervous system, have been transplanted to the central nervous system almost without fundamental modification of his basic concepts.

Some of us here today had the good fortune of knowing Dale personally. I believe that we were all impressed not only by his individual scientific achievements, but by the total impact of his personality, the sweep of his thought. It is perhaps the function of a meeting such as this to try to convey some of this impact to a younger generation to whom Dale represents only a name of a great scientist born 100 years ago.

(Received October 23, 1975)

References

BROWN, G.L., DALE, H.H. & FELDBERG, W. (1936). Chemical transmission of excitation from motor nerve to voluntary muscle. *J. Physiol., Lond.*, **87**, 42P.

BURN, J.H. & DALE, H.H. (1915). The action of certain quaternary ammonium bases. *J. Pharmac.*, **6**, 417–438.

DALE, H.H. & FELDBERG, W. (1934a). The chemical transmitter of vagus effects to the stomach. *J. Physiol., Lond.*, **81**, 320–334.

DALE, H.H. & FELDBERG, W. (1934b). The chemical transmitter of nervous stimuli to the sweat glands of the cat. *J. Physiol., Lond.*, **82**, 121–128.

DALE, H H., FELDBERG, W. & VOGT, M. (1936). Release of acetylcholine at voluntary nerve endings. *J. Physiol., Lond.*, **86**, 353–380.

FELDBERG, W. & GADDUM, J.H. (1934). The chemical transmitter at synapses in a sympathetic ganglion. *J. Physiol., Lond.*, **81**, 305–319.

FELDBERG, W., MINZ, B. & TSUTZIMURA, H. (1933). The mechanism of the nervous discharge of adrenaline. *J. Physiol., Lond.*, **80**, 15P.

Br. J. Pharmac. (1976), **56**, 9–19

ANTAGONISM OF γ-AMINOBUTYRIC ACID AND GLYCINE BY CONVULSANTS IN THE CUNEATE NUCLEUS OF CAT

R.G. HILL,[1] M.A. SIMMONDS & D.W. STRAUGHAN

Department of Pharmacology, The School of Pharmacy, University of London,
29/39 Brunswick Square, London WC1N 1AX

1 Some convulsant substances have been applied to single neurones in the cat cuneate nucleus by microiontophoresis. Numerical values were derived for the effectiveness and selectivity of the substances as antagonists of γ-aminobutyric acid (GABA) and glycine.

2 (+)-Bicuculline methochloride was the most effective GABA antagonist and it also excited many neurones. It antagonized GABA in 93% of experiments but also antagonized glycine in 41% of experiments. In most experiments the antagonism of GABA was greater than the antagonism of glycine resulting in an overall selective antagonism of GABA that was statistically significant. Nevertheless, in only about one quarter of the individual experiments was the GABA antagonism substantial and the selectivity clearcut.

3 (+)-Bicuculline and picrotoxin were less easily applied to neurones by microiontophoresis and were found to antagonize GABA in 30% and 35% of experiments, respectively. They also antagonized glycine in 25% and 30% of experiments, respectively. Overall, neither substance could be shown to be selective, statistically, although in the few individual experiments where the GABA antagonism was substantial the antagonism was clearly selective.

4 (+)-Tubocurarine antagonized GABA in 59% and glycine in 32% of experiments and it also excited many neurones. Penicillin antagonized GABA in 33% of experiments without antagonizing glycine. Neither antagonist caused any substantial antagonisms of GABA and neither showed significant selectivity overall. (−)-Bicuculline methochloride, leptazol and bemegride antagonized GABA or glycine in less than 10% of experiments, although (−)-bicuculline methochloride excited most neurones.

5 Strychnine antagonized glycine in every experiment while antagonizing GABA in only 5% of experiments. In each individual experiment the antagonism of glycine was substantial and clearly selective, resulting in a statistically significant selectivity overall.

6 It is concluded that the selective glycine antagonist strychnine is considerably better than the presently available GABA antagonists for distinguishing between responses to GABA and glycine, when the antagonists are applied by microiontophoresis.

Introduction

In an earlier paper (Hill, Simmonds & Straughan, 1973a), we compared a number of convulsant substances as antagonists of γ-aminobutyric acid (GABA) in the cerebral cortex of cats. We were unable to examine the selectivity of these antagonists in distinguishing between GABA and glycine-evoked depressions of neuronal firing because glycine had so little effect on cortical neurones. We have, therefore, made further experiments in the cuneate nucleus, an easily accessible area where the neurones can be readily depressed by both GABA and glycine.

Of the substances we have examined, strychnine is well known as an antagonist of glycine and it has frequently been reported that picrotoxin and (+)-bicuculline will antagonize GABA (e.g. Galindo, 1969; Bruggencate & Engberg, 1971; Curtis, Duggan, Felix, Johnston & McLennan, 1971a; Curtis, Duggan & Johnston, 1971b). (+)-Bicuculline methochloride and (+)-tubocurarine are two more recently introduced antagonists of GABA (Johnston, Beart, Curtis, Game, McCulloch & MacLachlan, 1972; Hill *et al.*, 1973a; Collins & Hill, 1974) and we have also used three other convulsants, penicillin, leptazol and bemegride, whose mechanisms of action are less well elucidated.

---

[1] Present address: Department of Pharmacology, University of Bristol, The Medical School, University Walk, Bristol BS8 1TD.

We have attempted to measure the effectiveness of these substances as antagonists of GABA and glycine and also to assign numerical values to their selectivity in distinguishing between the two agonists. To do this, we have used an analytical procedure (Hill & Simmonds, 1973; Simmonds, 1974) which is different from those used by previous investigators. It was hoped that such a study would allow comparison of the GABA receptors in the cuneate nucleus with those in the cerebral cortex and might also provide some clues to the mechanism of action of the convulsant substances. Furthermore, it has become clear that some quantitative comparisons of antagonist potency and selectivity *in vivo* are required to assist in the task of identifying GABA and glycine receptors *in vitro* (e.g. Young & Snyder, 1973; Zukin, Young & Snyder, 1974).

Methods

Surgical procedure

Experiments were made on a total of 26 cats of either sex weighing 2.0 to 3.5 kg and anaesthetized with 0.8 to 1.2% halothane in 70% N_2O and 30% O_2. Initially, the animals breathed spontaneously but, later, positive pressure artificial ventilation was sometimes applied, especially if neuronal firing became irregular. Rectal temperature was maintained at 37.5 ± 1°C.

The cat's head was mounted in a stereotaxic frame and flexed ventrally by 30°. A midline incision was made through the skin from the centre of the head down the nape of the neck. The cervical musculature was teased apart and the atlanto-occipital membrane exposed and removed. Since the head was flexed, a pressor foot (diameter 6 mm) could be placed centrally on the exposed area of medulla just caudal to the obex without removing the cerebellum. This substantially reduced movement of the underlying cuneate nucleus without any visible impairment of blood flow in the surface vessels. The pressor foot contained holes to allow the passage of microelectrodes. There was a continuous flow of c.s.f. over the surface of the tissue.

Experimental procedure

Seven-barrel glass micropipettes with tip diameters of 5–7 µm were prepared as described previously (Hill & Simmonds, 1973). Extracellular recordings of firing rate were made from neurones in the cuneate nucleus at depths of 0.6 to 2.6 mm from the surface of the medulla. No attempt was made to select neurones on the basis of their anatomical projections or firing patterns but all were depressed by GABA and glycine. Drugs were expelled from the micropipettes by iontophoresis and the experimental technique adopted was similar to that described before (Hill *et al.*, 1973a). Briefly, GABA and glycine were applied alternately with a period of 60 s elapsing between the end of one application and the beginning of the next. Each application was maintained until complete depression of neuronal firing was achieved, the expelling current being selected to give 50% reduction in firing rate in not less than 10 s and usually not more than 60 seconds. Once reproducible control responses were obtained, as characterized by the duration of amino acid application required to give 50% inhibition of firing (T_{50}), a 10–12 min continuous application of one of the convulsant drugs was made while the sequence of amino acid applications continued. Recovery from any effects of the drug was then followed.

Drugs

The drug solutions used in the pipettes were as follows: sodium DL-homocysteate (Calbiochem) or sodium L-glutamate (BDH), pH 8, 0.2 M; GABA (Sigma), pH 3.5, 0.2 M; glycine (BDH), pH 3.5, 0.2 M; (+)-bicuculline (K & K or Pierce Chemical), pH 3.5, 5 mM in 150 mM NaCl; strychnine sulphate (Hopkin & Williams); 5 mM in 150 mM NaCl; picrotoxin (Sigma), 5 mM in 150 mM NaCl; (+)-tubocurarine chloride (Burroughs Wellcome), 5 mM in 75 mM NaCl; (+)-bicuculline methochloride, 5 mM in 150 mM NaCl, and (–)-bicuculline methochloride 5 mM in 150 mM NaCl (both kindly prepared by Dr J.F. Collins, see Collins & Hill (1974)); leptazol (Emanuel), 1 M; bemegride (Nicholas), 30 mM in 150 mM NaCl; sodium benzyl penicillin (Crystapen-Glaxo), 0.5 M. Where no pH value is given, the pH of the drug solution was not adjusted.

All drugs were retained in the pipettes between periods of expulsion by appropriate retaining currents. For ionized drugs, 25 nA was usually sufficient although 40 nA was required for glycine. For drugs in NaCl solution, 10 or 15 nA was used.

Results

Recordings were obtained from 173 neurones. All the neurones were firing spontaneously and all were depressed by both GABA and glycine. Satisfactory studies with antagonists were completed on 89 neurones and more than one antagonist was studied on 42 of these. With nearly all neurones, the spontaneous firing rate was sufficiently high and steady for the study of depressant substances; on only eight neurones were continuous low currents of L-glutamate or DL-homocysteate used to increase the firing rates.

The currents of GABA and glycine generally found adequate were 20, 30 or 40 nA and, in most experiments, equal currents of the two amino acids

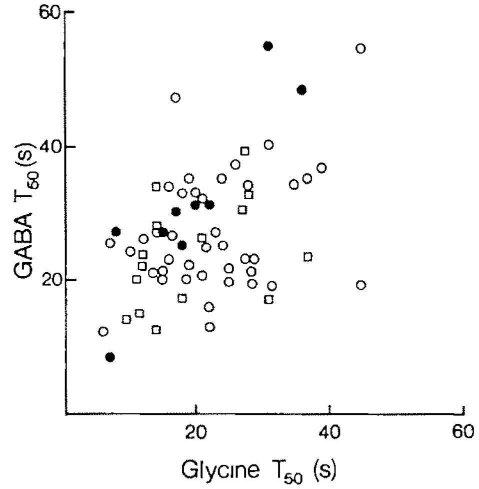


Figure 1 Comparison of the durations (s) of iontophoretic application of γ-aminobutyric acid (GABA) and glycine required to achieve 50% inhibition of neuronal firing (T_{50}). Each of the 64 points was obtained from a separate neurone The same currents were used to apply both GABA and glycine in individual experiments, the values being 20 nA (□), 30 nA (●) or 40 nA (○).

were used. For a representative sample of 64 neurones, the times taken to achieve 50% inhibition of firing (T_{50}) with GABA and glycine are correlated in Figure 1. There was no clear difference in the effectiveness of GABA and glycine in depressing these neurones. In eight experiments, the control periods of repeated alternate applications of GABA and glycine extended to 20 min or more and during this time the responses showed little variation in T_{50}. All the values for GABA fell within ±20% of the mean in each experiment and 95% of the values for glycine fell within ±25% of the mean. Thus, in the studies of antagonists, we have regarded changes in T_{50} values of less than 25% as 'no effect'.

A typical record of the effects of GABA and glycine on the firing rate of a neurone is shown in Figure 2(a). When the retaining current was removed from the strychnine barrel to allow strychnine to diffuse out, the depressant effect of glycine was progressively antagonized. This was apparent as a slowing of the time course of the glycine response indicating that a higher concentration of glycine was needed to achieve a given level of response (see Hill & Simmonds, 1973; Simmonds, 1974). Eventually, sufficient antagonism had occurred that a glycine expelling current of 30 nA was inadequate to suppress completely the firing of the neurone. At the same time, the responses to GABA were unaffected. The T_{50} value for each response was determined and the values are plotted against elapsed time for the entire experiment in Figure 2(b). The progressive antagonism of glycine by strychnine is clearly seen to be selective and, in other experiments where the strychnine application was maintained for longer, the glycine response was completely suppressed without the response to GABA being affected.

Only occasionally did the antagonists of GABA show such clearcut selectivity. One of our best examples is illustrated in Figure 3 where (+)-bicuculline methochloride reversibly antagonized GABA. Compared with the antagonism of glycine by strychnine, the degree of GABA antagonism by (+)-bicuculline methochloride always reached a plateau much more rapidly and the recovery from antagonism was generally much quicker. The greater the degree of GABA antagonism achieved, the more selective the antagonism appeared to be. The marked excitation caused by (+)-bicuculline methochloride was frequently seen but was not a necessary concomitant of GABA antagonism.

In order to compare the effectiveness of all the substances tested as antagonists of GABA and glycine, the degree of antagonism achieved in each experiment was expressed as T_{50} (test-control)/T_{50} (control) (see Hill & Simmonds, 1973). The T_{50} (test) values were obtained from the responses to GABA and glycine recorded 6–10 min after the start of a continuous application of the test substance. The T_{50} (control) values were means of values obtained just before the application of test substance and values obtained after at least 6 min recovery from the test substance. The value of T_{50} (test-control)/T_{50} (control) obtained for the displacement of the GABA response was plotted against the value obtained for displacement of the glycine response in the same experiment. The accumulated results obtained with (+)-bicuculline methochloride, (+)-bicuculline, picrotoxin, strychnine, (+)-tubocurarine, (–)-bicuculline methochloride, leptazol, bemegride and penicillin are shown in Figure 4. Values in the range –0.25 to +0.25 on the ordinate scale represent no effect on the GABA response and the same range of values on the abscissa scale represents no effect on the glycine response. Larger positive values indicate antagonism and negative values indicate potentiation.

It is apparent from these results that (+)-bicuculline methochloride (25–50 nA) was the most consistently effective GABA antagonist amongst the substances tested, antagonizing GABA in 93% of experiments. In nearly half of those experiments the antagonism was sufficient to give a value of T_{50} (test-control)/T_{50} (control) in excess of 1.0. The second most consistent GABA antagonist was (+)-tubocurarine (25–50 nA) (59% of experiments), but in no experiment was the antagonism sufficiently great to give a value in excess of 1.0. Next came picrotoxin (50–100 nA), penicillin (75–100 nA) and (+)-bicuculline (50–100 nA) which

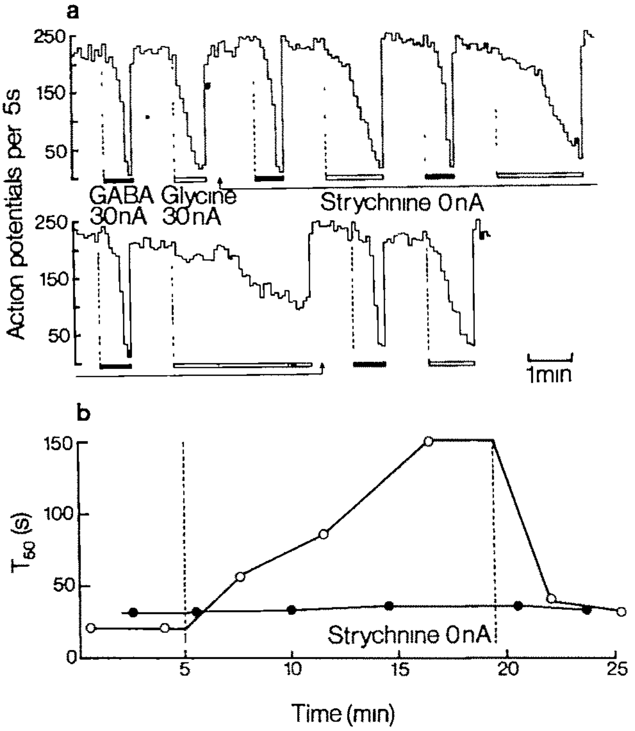


Figure 2 The selective antagonism of glycine by strychnine. (a) Histogram representation (5 s epochs) of the spontaneous firing rate of a neurone 1370 µm deep in the cuneate nucleus. Alternate micro-iontophoretic applications of γ-aminobutyric acid (GABA) 30 nA and glycine 30 nA were made throughout. Strychnine 0 nA was applied by removing the retaining current of −15 nA, thus allowing it to diffuse out of the pipette for the period indicated. (b) Time to 50% inhibition of neuronal firing (T_{50}) was measured for each response to GABA (●) and glycine (○) and plotted against elapsed time for the entire experiment.

antagonized GABA in 35%, 33% and 30% of experiments, respectively. In spite of these rather low figures, about one quarter of the picrotoxin antagonisms and two thirds of the (+)-bicuculline antagonisms had values in excess of 1.0. Strychnine (0–2 nA), (−)-bicuculline methochloride (25–50 nA), leptazol and bemegride (75–150 nA) were inactive with respect to antagonism of GABA.

As regards antagonism of glycine, it is clear that strychnine was the most consistently effective of the substances tested, antagonizing glycine in every experiment. In 95% of these experiments, the antagonism gave a value in excess of 1.0, in spite of the very low currents used. The second most consistent glycine antagonist was (+)-bicuculline methochloride (41% of experiments) but in only one sixth of those experiments did the antagonism give a value in excess of 1.0. Next came (+)-tubocurarine, picrotoxin and (+)-bicuculline which antagonized glycine in 32%, 30% and 25% of experiments, respectively. Nearly half of the (+)-tubocurarine antagonisms had values in excess of 1.0 but none of the picrotoxin or (+)-bicuculline antagonisms were over 1.0. Leptazol, bemegride, penicillin and (−)-bicuculline methochloride were inactive with respect to antagonism of glycine.

It is also interesting to note that (+)-bicuculline caused a considerable number of potentiations of GABA and glycine, as it did of GABA in the cerebral cortex (Hill *et al.*, 1973a).

The difference between the value of T_{50} (test-control)/T_{50} (control) obtained for GABA and that obtained for glycine in each experiment was taken as a measure of the selectivity of the test substance in antagonizing GABA or glycine. The greater the difference between the values, the greater was the selectivity demonstrated to be. In Table 1, these measures of selectivity are divided into seven consecutive ranges and the numbers of experiments falling into each range are shown. To determine whether any of the test substances demonstrated statistically significant selectivity overall in

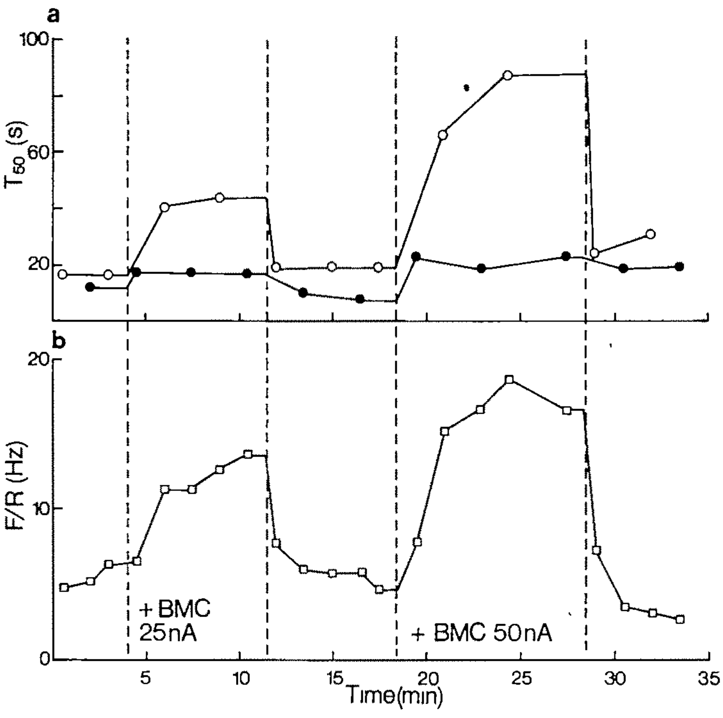


Figure 3 The selective antagonism of γ-aminobutyric acid (GABA) by (+)-bicuculline methochloride (BMC). (a) Shows values of T_{50} obtained from alternate microiontophoretic applications of GABA 40 nA (○) and glycine 40 nA (●). These results were obtained from a spontaneously firing neurone, 951 µm deep in the cuneate nucleus. (b) Shows the baseline firing rate of the neurone during the 30 s before each application of GABA or glycine. The values are plotted against elapsed time throughout the experiment.

antagonizing either GABA or glycine, the results were examined by the Wilcoxon matched-pairs signed-rank test. As indicated in Table 1, (+)-bicuculline methochloride was shown to antagonize GABA selectively rather than glycine, and strychnine to antagonize glycine selectively rather than GABA. None of the other test substances showed significant selectivity overall in their antagonism of GABA and glycine. However, it should be pointed out that in the individual experiments where picrotoxin and (+)-bicuculline antagonized GABA with values of T_{50} (test-control)/T_{50} (control) in excess of 1.0, the antagonisms could all be described as selective, since the value for GABA antagonism was always substantially greater than that for glycine antagonism. Similarly, in the individual experiments where (+)-tubocurarine antagonized glycine with values of T_{50} (test-control)/T_{50} (control) in excess of 1.0, the antagonisms could all be described as selective.

In some experiments, the test substances themselves changed the firing rates of the neurones in a reversible manner. To examine whether these effects were related to the ability of the test substance to antagonize GABA or glycine, values of T_{50} (test-control)/T_{50} (control) were plotted against the simultaneous effects on the overall background firing rates of the neurones (Figure 5). The most marked excitant effects were caused by (+)-bicuculline methochloride and (+)-tubocurarine; (—)-bicuculline methochloride was moderately excitant but no consistent excitant or depressant effects were seen with the remaining substances. With none of the test substances, however, was there any significant correlation between their displacement of background firing rate and their effects on responses to GABA or glycine.

The currents used to expel picrotoxin, (+)-bicuculline, leptazol, bemegride and penicillin were as high as could be applied without an excessive incidence of unacceptably noisy recordings and failure of the drug barrel to pass a steady current. Further prolongation of the drug applications would have had little effect since, after 10 min continuous application, the concentration of drug around the neurone must have come close to the asymptotic concentration for the current being passed. The lower currents used to expel (+)-bicuculline methochloride, (—)-bicuculline

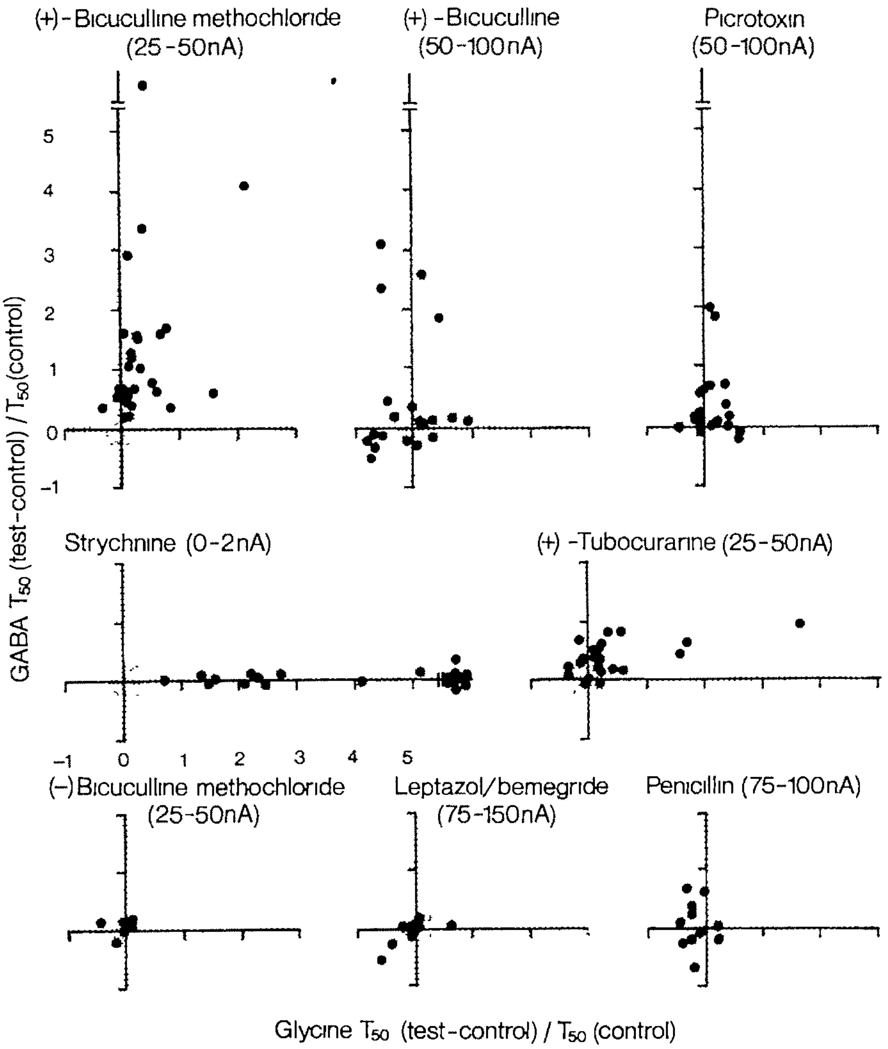


Figure 4 Effects of some convulsant substances on responses of neurones in the cuneate nucleus to γ-aminobutyric acid (GABA) and glycine. The sizes of the effects are expressed as T_{50} (test-control)/T_{50} (control) for GABA (ordinate scale) and glycine (abscissa scale). Each point represents a separate experiment Negative values indicate potentiation and positive values antagonism The stippled areas indicate the ±0.25 no effect zones. In some experiments, the antagonisms were sufficiently large that GABA or glycine, in the currents used, became unable to depress neuronal firing to 50%; in these cases, the point is plotted at the far end of the scale

methochloride and (+)-tubocurarine reflect the limitation which the marked excitatory actions of these substances placed on the amounts that could be applied to neurones. On the other hand, the currents used to expel strychnine were deliberately kept to a low level. When higher currents of strychnine in the range 25–50 nA were applied to six neurones, the responses to glycine were completely suppressed while the responses to GABA were antagonized in 4 of the experiments with values of T_{50} (test-control)/T_{50} (control) up to 1.20.

Discussion

From each experiment we have derived numerical values for the antagonism of GABA and glycine by one of the test substances. This has enabled us to make overall assessments of how consistently the antagonisms occurred with each substance and to make some objective comment on the magnitudes of the antagonisms observed. We have also examined the selectivity of each antagonist in distinguishing between GABA and glycine. Detailed descriptions of the analytical method we have used are given elsewhere (Hill & Simmonds, 1973; Simmonds, 1974) as, also, are previous examples of its use in evaluating the antagonism of both inhibitory and excitatory substances (Hill *et al.*, 1973a; Clarke, Forrester & Straughan, 1974). This, however, is the first time that the method has been extended to measure selectivity of antagonism.

In the interpretation of our results and drawing conclusions from them we have been well aware of the limitations of microiontophoretic methodology. The important assumptions on which our own methods were based include: (i) a progressively increasing rate of release of agonist during a constant current application, rather than a steady rate of release, resulting from dilution of the active ion in the pipette tip during prior passage of retaining currents (Hill & Simmonds, 1973; Clarke, Hill & Simmonds, 1973); (ii) equating T_{50} with concentration of agonist required to produce a given degree of receptor occupation which, in turn, depends on the relationship between response and receptor occupation being unaffected by

Table 1 The selectivity of some convulsants as antagonists of γ-aminobutyric acid (GABA) or glycine

	Numbers of experiments in which the difference between T_{50} (test-control)/T_{50} (control) values for GABA and glycine were:							Overall selective antagonist of·
	GABA > glycine				*glycine > GABA*			
	>2.0	*1.0–2.0*	*0.5–1.0*	*<0.5**	*0 5–1.0*	*1.0–2.0*	*>2 0*	
(+)-Bicuculline methochloride (25–50 nA)	3	5	10	9	1	1	0	GABA (*P*<0.001)
(+)-Bicuculline (50–100 nA)	3	1	1	12	3	0	0	N S.
Picrotoxin (50–100 nA)	0	2	3	13	2	0	0	N S.
Strychnine (0–2 nA)	0	0	0	0	1	3	16	glycine (*P*<0 001)
(+)-Tubocurarine (25–50 nA)	0	0	1	18	0	3	0	N.S.
(–)-Bicuculline methochloride (25–50 nA)	0	0	0	8	0	0	0	N.S.
Leptazol/bemegride (75–150 nA)	0	0	0	11	1	0	0	N.S
Penicillin (75–100 nA)	0	0	3	9	0	0	0	N.S.

* Includes all experiments where both GABA and glycine values were less than 0.25.

Table 2 Comparison of the frequency of γ-aminobutyric acid (GABA) antagonism by some convulsant substances applied by microiontophoresis onto single neurones in the cuneate nucleus and cerebral cortex

	*Cuneate nucleus**	*Cerebral cortex†*
(+)-Tubocurarine	59%	85%
Picrotoxin	35%	68%
Penicillin	33%	48%
(+)-Bicuculline	30%	31%
Leptazol/bemegride	0	20%

* Present results † Taken from Hill *et al.*, 1973a.

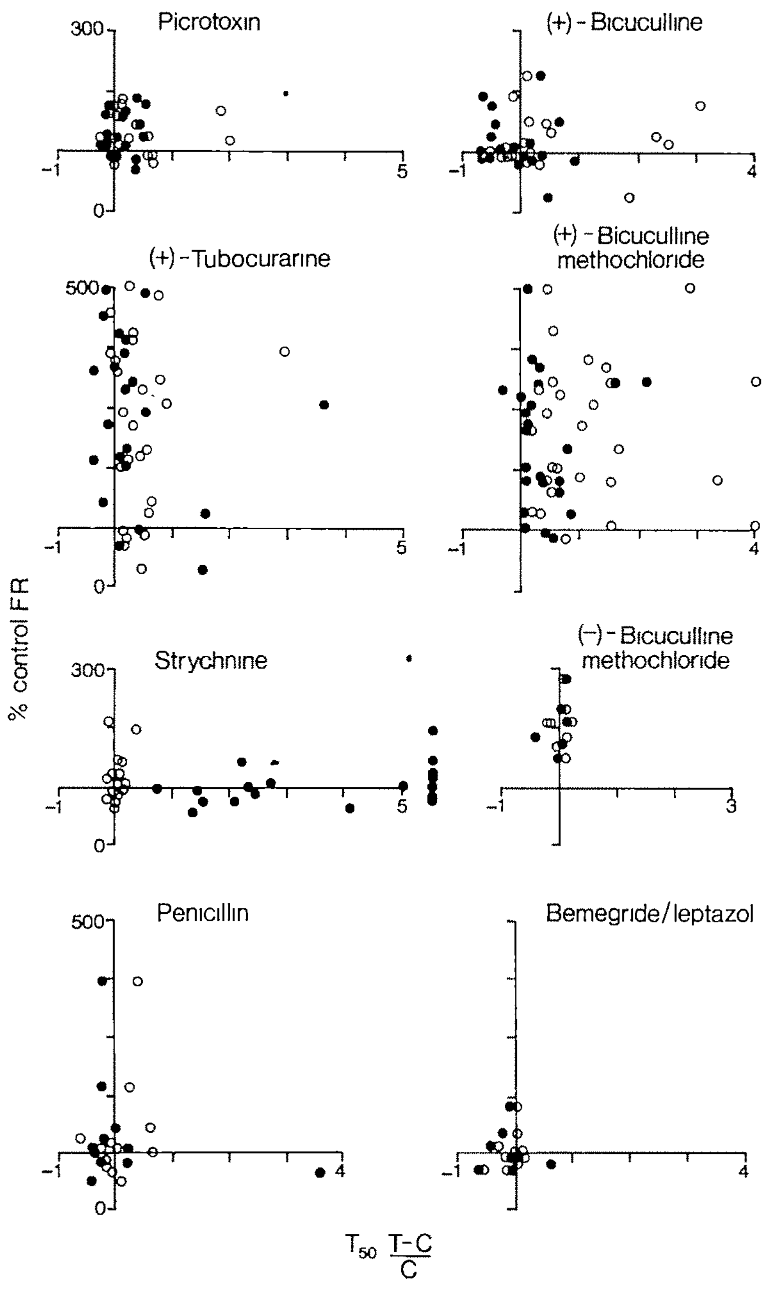


Figure 5 Relationship between effects on responses to γ-aminobutyric acid (GABA) and glycine and effects on background firing rate (FR) of some convulsant substances applied to neurones in the cuneate nucleus. The data are drawn from the same experiments contributing to Figure 4 and the iontophoretic currents used to apply the convulsants are the same as in that figure. The effects of the convulsants on the responses to GABA (○) and glycine (●) are expressed as T_{50} (test-control)/T_{50} (control) on the horizontal axes. The effects of the convulsants on background firing rate are indicated on the vertical axes as % control and were calculated from the mean values of neuronal firing during the 30 s prior to each application of GABA or glycine. With none of the convulsants was there any significant correlation between effects on background firing rate and effects on responses to GABA or glycine.

desensitization over different time intervals; (iii) minimal dependence of the numerical values of antagonism on the precise sizes of the agonist expelling and retaining current (Hill & Simmonds, 1973). Although we believe these to be reasonable assumptions, there is a possibility that they may not always hold good. When comparing the interpretations and conclusions of different authors, therefore, it is important to take account of the assumptions on which their respective methodologies are based.

Six of the substances tested as antagonists in the present experiments had also been tested previously on cortical neurones (Hill *et al.*, 1973a). In terms of how consistently they antagonized GABA with values of T_{50} (test-control)/T_{50} (control) in excess of 0.25, all six substances appeared in the same rank order in both the cuneate nucleus and the cerebral cortex (in descending order of consistency: (+)-tubocurarine, picrotoxin, penicillin, (+)-bicuculline, leptazol and bemegride) (Table 2). Nevertheless, with all except (+)-bicuculline, there was a greater percentage of 'no effects' in the cuneate nucleus. A similar difference between cuneate nucleus and cortex is apparent in terms of the magnitude of the antagonisms observed; thus, (+)-bicuculline was approximately equally effective as a GABA antagonist in both areas of brain while the other five substances caused generally smaller antagonisms in the cuneate nucleus, this being particularly marked with (+)-tubocurarine.

To what extent these differences may be due to less efficient iontophoretic release of substances in the present experiments is not clear. There is, certainly, no support for such an explanation from a comparison of the incidence of direct effects of the test substances on neuronal firing rate in the two series of experiments. An alternative explanation could involve the possibly different distributions of GABA receptors at pre- and post-synaptic sites in cuneate nucleus and cerebral cortex. Both pre- and post-synaptic inhibition have been demonstrated in the cuneate nucleus (Andersen, Etholm & Gordon, 1970) and GABA has been suggested as the transmitter for both mechanisms (Banna & Jabbur, 1969; Davidson & Reisine, 1971; Kelly & Renaud, 1971; Banna, Naccache & Jabbur, 1972; Kelly & Renaud, 1973c). In the cortex, however, only a post-synaptic action of GABA has been shown to occur, as yet (Krnjević & Schwartz, 1968).

It may be relevant that all the substances found capable of antagonizing GABA have been shown to reduce the P-wave or dorsal root potential correlates of pre-synaptic inhibition (Banna & Jabbur, 1969; Banna *et al.*, 1972; Davidoff, 1972; Hill, Simmonds & Straughan, 1973c, 1974). But other substances such as leptazol and bemegride can reduce pre-synaptic inhibition (Banna & Jabbur, 1970; Hill *et al.*, 1974) although we have been unable to show that either substance can antagonize GABA in the cuneate nucleus or the cerebral cortex (Hill *et al.*, 1973a). Even in concentrations up to 5×10^{-3} M, neither leptazol nor bemegride will antagonize the actions of GABA on the isolated superior cervical ganglion of the rat (N.G. Bowery, personal communication; see Bowery & Brown, 1974). Thus, the ability of a substance to reduce pre-synaptic inhibition does not necessarily indicate that it is a GABA antagonist. Similarly, the convulsant properties of leptazol and bemegride cannot yet be related to antagonism of GABA.

An important part of the present work has been concerned with the assessment of selectivity of an antagonist. Although numerical analyses are innately more objective than the simple visual inspection of neuronal firing rate records, care must still be exercised in the interpretation of selectivity measurements. For instance, a high degree of selectivity can only be demonstrated if the antagonist is sufficiently potent to cause a large displacement of the agonist-response curve. On the other hand, a low degree of selectivity which occurs very consistently may prove to be statistically significant but the selectivity might be too low to make practical use of the antagonist in identifying a specific receptor. When an antagonist is consistently effective, potent and its selectivity is clearcut, as in the antagonism of glycine by strychnine, no difficulty arises and there is virtually no disagreement between different authors. Descriptions attached to the antagonists of GABA by different authors, however, are more controversial, e.g. compare Curtis, Duggan, Felix & Johnston (1970a, b), Curtis *et al.* (1971a) and Dreifuss & Matthews (1972) with Godfraind, Krnjević & Pumain (1970), Bruggencate & Sonnhof (1972), Tebecis (1973), Hill, Simmonds & Straughan (1973b) and the present results. These differences are probably due to the antagonism not being sufficiently potent and selective to be seen clearly in all experimental situations.

In the present experiments, (+)-bicuculline methochloride was the only GABA antagonist that could be shown to distinguish overall between responses to GABA and those to glycine in a statistical test. Even so, while several of the individual experimental results showed clearcut selectivity, in several other experiments the responses to GABA were only slightly more antagonized than responses to glycine and some showed a reversed selectivity. (+)-Bicuculline and picrotoxin did not prove to be selective overall as GABA antagonists in the statistical test, even though, in the several individual experiments where GABA was substantially antagonized, the antagonism was clearly selective. It is possible that the failure of these two substances to show statistically significant selectivity overall was due to the small size of many of the GABA antagonisms rather than to an intrinsically poor selectivity. Similarly, the high proportion of small antagonisms of GABA by (+)-tubocurarine did not prove to be significant overall; indeed, there were occasional clearcut selective antagonisms of glycine.

This is in keeping with a report that (+)-tubocurarine can antagonize both GABA and glycine on spinal cord neurones (Curtis, Game & McCulloch, 1974).

Rather different conclusions about the selectivity of (+)-bicuculline and picrotoxin as GABA antagonists in the cuneate nucleus were reached by Kelly & Renaud (1973a, b). Their experimental design and method of quantitative analysis were different from our own and may have allowed the reported selectivity towards GABA to become more apparent. For example, Kelly & Renaud (1973a) observed that 'Although in general, bicuculline seldom caused a significant shift of the glycine log-current response curve, many of our records showed bicuculline to slow the onset of the response to glycine'. This was interpreted by the authors to mean that (+)-bicuculline did not antagonize glycine. In contrast, the method of analysis we have used attempts to quantify such slowing of responses as an essential characteristic of antagonism. Thus, the apparent selectivity of these substances as GABA antagonists depends upon the precise way in which the experiments are performed and the results analysed and interpreted.

From all these considerations of our present results, we conclude that (+)-bicuculline methochloride is the substance most likely to antagonize GABA substantially and selectively when the antagonists are applied by microiontophoresis. Its greater solubility compared with (+)-bicuculline or picrotoxin and easier ejection by iontophoresis may, in part, account for its superiority over these substances. It does, however, have some different properties from (+)-bicuculline and should not, therefore, be regarded simply as a more soluble form of (+)-bicuculline. In particular, the excitant properties of (+)-bicuculline methochloride may cause complications in its use. However, in the present experiments there was no correlation between excitation and GABA antagonism and, as a further control, the (−)-isomer can be compared since it does not antagonize GABA but retains an ability to excite neurones. Nevertheless, when this minor reservation is coupled with the larger problem of (+)-bicuculline methochloride causing some antagonisms of glycine, it becomes clear that the best means of separating GABA-operated from glycine-operated pathways at present is the use of strychnine. The currently available GABA antagonists may provide corroborative evidence but their use cannot stand alone. Despite this limitation, careful measurement of the effectiveness and selectivity of all substances capable of antagonizing GABA or glycine contributes to our understanding of inhibitory processes and to more detailed characterization of the GABA and glycine receptors.

We thank Mr C.J. Courtice, Mr T. Gonye and Mrs B. Richards-Pugh for skilled technical assistance. This work was supported by a Programme Grant from the M.R.C. to D.W.S. and by an equipment grant from The Wellcome Trust. R.G.H. was a Wellcome Research Student.

References

ANDERSEN, P., ETHOLM, B. & GORDON, G. (1970). Presynaptic and postsynaptic inhibition elicited in the cat's dorsal column nuclei by mechanical stimulation of the skin. *J. Physiol.*, **210**, 433–455.

BANNA, N.R. & JABBUR, S.J. (1969). Pharmacological studies on inhibition in the cuneate nucleus of the cat. *Int. J. Neuropharmac.*, **8**, 299–307.

BANNA, N.R. & JABBUR, S.J. (1970). The action of bemegride on presynaptic inhibition. *Neuropharmacology*, **9**, 553–560.

BANNA, N.R., NACCACHE, A. & JABBUR, S.J. (1972). Picrotoxin-like action of bicuculline. *Eur. J. Pharmac.*, **17**, 301–302.

BOWERY, N.G. & BROWN, D.A. (1974). Depolarizing actions of γ-aminobutyric acid and related compounds on rat superior cervical ganglia in vitro. *Br. J. Pharmac.*, **50**, 205–218.

BRUGGENCATE, G. TEN & ENGBERG, I. (1971). Iontophoretic studies in Deiter's nucleus of the inhibitory actions of GABA and related amino acids and the interactions of strychnine and picrotoxin. *Brain Res.*, **25**, 431–448.

BRUGGENCATE, G. TEN & SONNHOF, U. (1972). Effects of glycine and GABA and blocking actions of strychnine and picrotoxin in the hypoglossus nucleus. *Pflügers Arch.*, **334**, 240–252.

CLARKE, G., FORRESTER, P.A. & STRAUGHAN, D.W. (1974). A quantitative analysis of the excitation of single cortical neurones by acetylcholine and L-glutamic acid applied microiontophoretically. *Neuropharmacology*, **13**, 1047–1055.

CLARKE, G., HILL, R.G. & SIMMONDS, M.A. (1973). Microiontophoretic release of drugs from micropipettes: use of ^{24}Na as a model. *Br. J. Pharmac.*, **48**, 156–161.

COLLINS, J.F. & HILL, R.G. (1974). (+) and (−) bicuculline methochloride as optical isomers of a GABA antagonist. *Nature, Lond.*, **249**, 845–847.

CURTIS, D.R., DUGGAN, A.W., FELIX, D. & JOHNSTON, G.A.R (1970a). GABA, bucuculline and central inhibition. *Nature, Lond.*, **226**, 1222–1224.

CURTIS, D.R., DUGGAN, A.W., FELIX, D. & JOHNSTON, G.A.R. (1970b). Bicuculline and central GABA receptors. *Nature, Lond.*, **228**, 676–677.

CURTIS, D.R., DUGGAN, A.W., FELIX, D., JOHNSTON, G.A.R & McLENNAN, H. (1971a). Antagonism between bicuculline and GABA in the cat brain. *Brain Res.*, **33**, 57–73.

CURTIS, D.R., DUGGAN, A.W. & JOHNSTON, G.A.R. (1971b). The specificity of strychnine as a glycine antagonist in the mammalian spinal cord. *Expl Brain Res.*, **12**, 547–565.

CURTIS, D.R., GAME, C.J.A. & McCULLOCH, R.M. (1974).

Antagonism of inhibitory amino acid action by tubocurarine. *Br. J. Pharmac.*, **52**, 101–103.

DAVIDOFF, R.A. (1972). Penicillin and presynaptic inhibition in the amphibian spinal cord. *Brain Res.*, **36**, 218–222.

DAVIDSON, N. & REISINE, H. (1971). Presynaptic inhibition in cuneate blocked by GABA antagonists. *Nature New Biol.*, **234**, 223–224.

DREIFUSS, J.J. & MATTHEWS, E.K. (1972). Antagonism between strychnine and glycine, and bicuculline and GABA in the ventromedial hypothalamus. *Brain Res.*, **45**, 599–603.

GALINDO, A. (1969). GABA-picrotoxin interaction in the mammalian central nervous system. *Brain Res.*, **14**, 763–767.

GODFRAIND, J.M., KRNJEVIĆ, K. & PUMAIN, R. (1970). Doubtful value of bicuculline as a specific antagonist of GABA. *Nature, Lond.*, **228**, 675–676.

HILL, R.G. & SIMMONDS, M.A. (1973). A method for comparing the potencies of γ-aminobutyric acid antagonists on single cortical neurones using microiontophoretic techniques. *Br. J. Pharmac.*, **48**, 1–11.

HILL, R.G., SIMMONDS, M.A. & STRAUGHAN, D.W. (1973a). A comparative study of some convulsant substances as γ-aminobutyric acid antagonists in the feline cerebral cortex. *Br. J. Pharmac.*, **49**, 37–51.

HILL, R.G., SIMMONDS, M.A. & STRAUGHAN, D.W. (1973b). Amino acid antagonists and the depression of cuneate neurones by γ-aminobutyric acid (GABA) and glycine. *Br. J. Pharmac.*, **47**, 642–643P.

HILL, R.G., SIMMONDS, M.A. & STRAUGHAN, D.W (1973c). Presynaptic inhibition and the depressant actions of GABA and glycine in the feline cuneate nucleus: changes related to electrographic seizure activity. *J. Physiol.*, **234**, 83–84P.

HILL, R.G., SIMMONDS, M.A. & STRAUGHAN, D.W. (1974). Convulsant substances as antagonists of GABA and presynaptic inhibition in the cuneate nucleus. *Br. J. Pharmac.*, **52**, 117P.

JOHNSTON, G.A.R., BEART, P.M., CURTIS, D.R., GAME, C.J.A., McCULLOCH, R.M. & MACLACHLAN, R.M. (1972). Bicuculline methochloride as a GABA antagonist. *Nature, New Biol.*, **240**, 219–220.

KELLY, J.S. & RENAUD, L.P. (1971). Postsynaptic inhibition in the cuneate blocked by GABA antagonist. *Nature, New Biol.*, **232**, 25–26.

KELLY, J.S. & RENAUD, L.P. (1973a). On the pharmacology of the γ-aminobutyric acid receptors on the cuneo-thalamic relay cells of the cat. *Br. J. Pharmac.*, **48**, 369–386.

KELLY, J.S. & RENAUD, L.P. (1973b). On the pharmacology of the glycine receptors on the cuneo-thalamic relay cells in the cat. *Br. J. Pharmac.*, **48**, 387–395.

KELLY, J.S. & RENAUD, L.P. (1973c). On the pharmacology of ascending, descending and recurrent postsynaptic inhibition of the cuneo-thalamic relay cells in the cat. *Br. J. Pharmac.*, **48**, 396–408.

KRNJEVIĆ, K. & SCHWARTZ, S. (1968). The inhibitory transmitter in the cerebral cortex. In *Structure and functions of inhibitory neuronal mechanisms*, pp. 419–427. Oxford: Pergamon.

SIMMONDS, M.A. (1974). Quantitative evaluation of responses to microiontophoretically applied drugs. *Neuropharmacology*, **13**, 401–406.

TEBECIS, A.K. (1973). Transmitters and reticulospinal neurons. *Expl Neurol.*, **40**, 297–308.

YOUNG, A.B & SNYDER, S.H. (1973). Strychnine binding associated with glycine receptors of the central nervous system. *Proc. natn. Acad. Sci. U.S.A.*, **70**, 2832–2836.

ZUKIN, S.R., YOUNG, A.B. & SNYDER, S.H (1974). Gamma-aminobutyric acid binding to receptor sites in the rat central nervous system. *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4802–4807.

(Received July 17, 1974,
Revised April 9, 1975.)

Br. J. Pharmac. (1976), **56**, 21–24

ANTIOXOTREMORINE ACTION OF PROPRANOLOL

D.K. GANGULY
Indian Institute of Experimental Medicine, 4, Raja Subodh Mullick Road,
Jadavpur, Calcutta-32, India

1 The influence of propranolol on the neuromuscular, tremor-producing and muscarinic effects of oxotremorine was examined.

2 In the isolated rat phrenic nerve-diaphragm preparation the neuromuscular blocking effect of oxotremorine was inhibited by propranolol in a dose-dependent manner.

3 Propranolol intensified the paralytic effect of tubocurarine in the rat diaphragm and prevented antagonism of tubocurarine paralysis by tetraethylammonium.

4 Propranolol was devoid of any curare-like effect in the isolated rectus abdominis muscle of the frog.

5 Vasodepressor responses to oxotremorine in rats and spasmogenic responses to oxotremorine in guinea-pig ileum were not antagonized by propranolol.

6 A dose-dependent antagonism of oxotremorine-induced tremor in mice was observed after pretreatment with propranolol and it is suggested that this effect is due to an antagonism of a presynaptic effect of oxotremorine at skeletal neuromuscular junctions.

Introduction

Propranolol diminishes tremor in patients with Parkinson's disease (Owen & Marsden, 1965; Marsden, Meadows, Lange & Watson, 1967) and inhibits oxotremorine-induced tremor in mice and rats (Jacobi, 1957; Agarwal & Bose, 1967; Cox & Potkonjak, 1970). Some other central effects of oxotremorine, including its analgesic and hypothermic effects are not affected by propranolol (Hermansen, 1968) and it has therefore been suggested that the anti-tremor action of propranolol is mediated peripherally (Marsden *et al.*, 1967). However, the effect does not appear to be due to blockade of β-adrenoceptors (Agarwal & Bose, 1967).

Oxotremorine is known to affect skeletal neuromuscular sites (Csillik, 1964; Elmqvist & McIssac, 1967; Ganguly & Chaudhuri, 1970) and produces a nicotinic vasopressor response in atropinized rats (Ganguly & Saha, 1972) and this paper presents evidence for a neuromuscular site of action for the anti-tremor effect of propranolol on oxotremorine-induced tremor.

Methods

Isolated phrenic nerve-diaphragm preparations were prepared from albino rats (150 to 200 g) of either sex, according to the method of Bülbring (1946). The preparations were suspended in a 40 ml bath containing physiological solution of the following composition (g/l): NaCl 8, KCl 0.2, $CaCl_2$ 0.2, $NaHCO_3$ 1, dextrose 2, NaH_2PO_4 0.05, $MgCl_2$ 0.01 bubbled with oxygen at 31°C. Muscle contractions were recorded with a simple straw lever of minimum load giving 8-fold magnification. The phrenic nerve was stimulated at 0.2 to 0.3 Hz with supramaximal rectangular pulses of 0.2 ms duration.

The rectus abdominis muscle of the frog was prepared in the usual way using a 10 ml bath containing frog-Ringer solution (g/l): NaCl 6.0, KCl 0.075, $CaCl_2$ 0.1, $NaHCO_3$ 1.0 bubbled with air.

Blood pressure effects were examined in male albino rats (150 to 230 g) anaesthetized with urethane (1.4 g/kg s.c.). After inserting cannulae into the left carotid artery and the left femoral vein, heparin was administered (100 u i.v.) and the animal was artificially ventilated. Blood pressure was recorded using a mercury manometer.

Guinea-pig isolated ileum was set up in Tyrode solution (g/l): NaCl 8, KCl 0.2, $NaHCO_3$ 1, dextrose 1, NaH_2PO_4 0.05, $MgCl_2$ 0.01 at 37°C.

Ten minutes after intraperitoneal injection of oxotremorine (250 mg/kg), tremor was assessed in groups of 10 male mice (20–25 g) by visual scoring and by using an objective scoring device. Propranolol (1, 2 and 4 mg/kg i.p.) was given to some groups 1 h before

P 25830

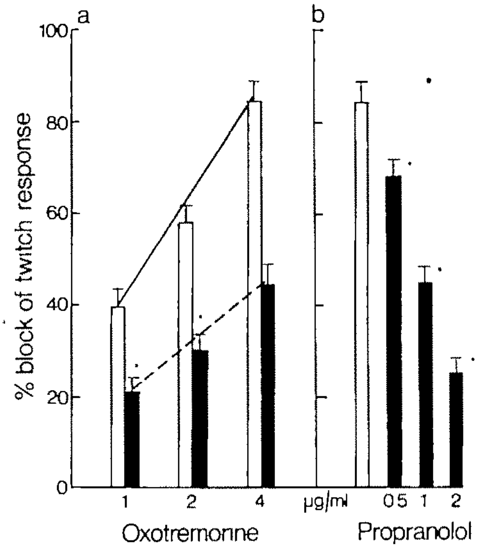


Figure 1 The percentage blockade of the twitch response of stimulated rat diaphragm caused by oxotremorine alone (open columns) and oxotremorine together with propranolol (closed columns). In (a) the concentration of propranolol was 1 μg/ml and that of oxotremorine was varied. In (b) the concentration of oxotremorine was 4 μg/ml and that of propranolol was varied. Vertical lines represent s e. * indicates a significant difference in the oxotremorine effect caused by addition of propranolol ($P<0.05$).

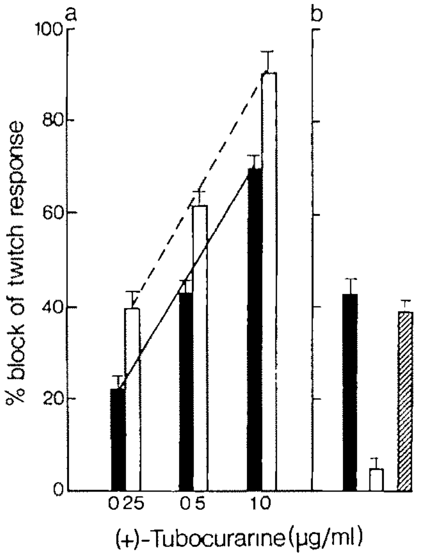


Figure 2 The percentage blockade of the twitch response of indirectly stimulated rat diaphragm caused in (a) by (+)-tubocurarine (0.25, 0.5 and 0.1 μg/ml) alone (closed columns) and after 5 min incubation with 1 μg/ml propranolol (open columns) and in (b) by (+)-tubocurarine (0 1 μg/ml) alone (closed columns) and after 5 min incubation with 50 μg/ml TEA (open column) or 50 μg/ml TEA and 1 μg/ml propranolol (hatched column) Vertical lines represent s.e.

oxotremorine. Scores were assessed visually by the method of Spencer (1965); no tremor = 0, moderate or intermittent tremor = 1.0 and pronounced continuous tremor = 2.0. A permanent record of tremor was obtained by a modification of the method described by Ahmed & Taylor (1959). A perforated plastic soap box (114 mm × 63 mm × 37 mm) was suspended by a stainless steel wire from an iron stand. A thin steel nozzle (length = 25 mm), permanently fixed to one side of the plastic box, was attached to a piezoelectric transducer connected to the preamplifying channel (model R 67z) of a Gallileo 3-channel ink-writing polygraph to obtain a record of tremor. The plastic box, stand and transducer were placed in a rectangular glass case to reduce environmental disturbance. Mice were acclimatized by placing them individually in the box for 10 to 15 min before each experiment. Recordings were made before and 10 min after injecting oxotremorine.

The following drugs were used: oxotremorine sesquifumarate (Aldrich), (±)-propranolol hydrochloride (I.C.I.), (+)-tubocurarine chloride (Sigma), acetylcholine chloride (E. Merck), tetraethylammonium bromide (Fluka).

Results

Isolated rat phrenic nerve-diaphragm

Twitch responses of indirectly stimulated rat diaphragm preparation were unaffected within 15 min of adding propranolol alone at concentrations of up to 5 μg/ml but prior incubation with propranolol for 5 min markedly reduced the neuromuscular blocking effect of oxotremorine (Figure 1).

In another series of experiments, interactions of propranolol with (+)-tubocurarine and tetraethylammonium (TEA) were studied. TEA is known to antagonize the paralytic effect of (+)-tubocurarine by increasing the release of acetylcholine at neuromuscular junctions (Bowman, Hemsworth & Rand, 1962; Collier & Exley, 1963). Prior incubation with propranolol for 5 min intensified the paralytic effect of tubocurarine (Figure 2). Previous incubation with TEA (50 to 100 μg/ml) for 5 min significantly reduced the effect of (+)-tubocurarine (0.5 μg/ml) and this antagonistic effect of TEA was almost lost in the presence of propranolol (1 μg/ml; Figure 2). A typical experiment is illustrated in Figure 3.

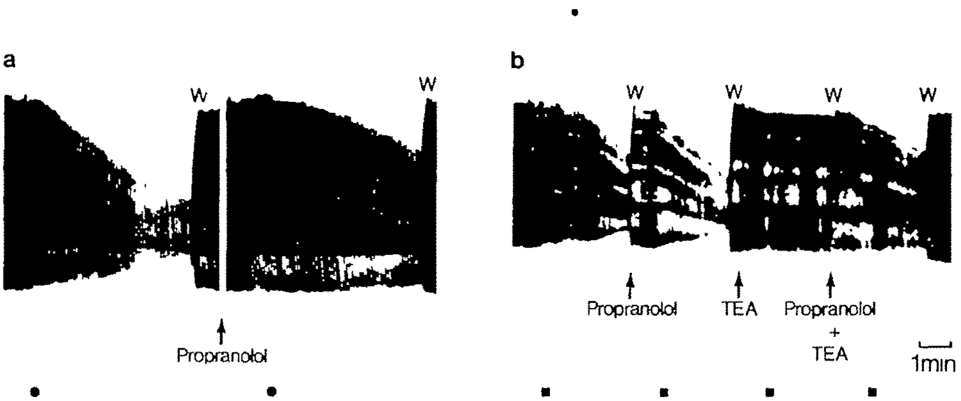


Figure 3 Tracings of twitch responses of indirectly stimulated rat diaphragm (a) The effect of 2 µg/ml oxotremorine (●) alone and 5 min after incubating the diaphragm with propranolol (1 µg/ml). (b) The effect of 0.5 µg/ml (+)-tubocurarine (■) alone and at 5 min after incubation with propranolol (1 µg/ml), TEA (100 µg/ml) or both. w=wash.

Frog isolated rectus abdominis

The influence of propranolol on postsynaptic skeletal muscle receptors was examined by using the frog isolated rectus abdominis preparation. Propranolol, up to a concentration of 5 µg/ml, failed to modify the responses to exogenous acetylcholine (4 experiments). Moreover, the inhibitory effect of (+)-tubocurarine on responses to exogenous acetylcholine remained unaffected in the presence of propranolol (5 experiments).

Muscarinic responses to oxotremorine

Vasodepressor responses to oxotremorine were not significantly affected when repeated 30 min after propranolol (2 mg/kg i.v.; 5 experiments). Similarly the vasodepressor responses to acetylcholine were unchanged after giving propranolol (5 experiments).

Spasmogenic responses of guinea-pig isolated ileum to oxotremorine (0.01 µg/ml) and acetylcholine (0.01 µg/ml) were not altered in the presence of propranolol (1 µg/ml; 4 experiments).

Tremor responses to oxotremorine

Pretreatment with propranolol (1, 2 and 4 mg/kg i.p.) markedly reduced oxotremorine-tremor in mice. In the studies reported in Figure 4 tremor was assessed visually but confirmation was obtained using objective measurement (see **Methods**).

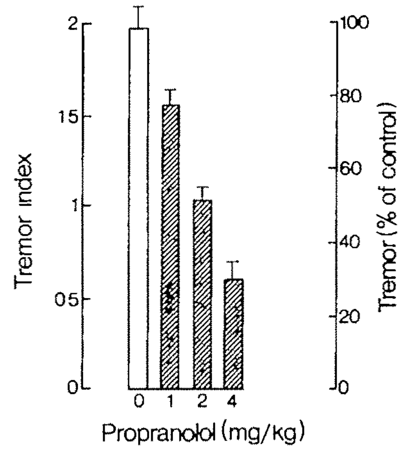


Figure 4 The tremor index of mice given oxotremorine (250 µg/kg i.p.): open columns, controls; hatched columns, at 1 h after propranolol (1, 2 and 4 mg/kg i.p.). Mean score for groups of 10 mice and the percentage of control values. Vertical lines represent s.e.

Discussion

The present experiments confirm the anti-tremor action of propranolol reported by others (Jacobi, 1957; Agarwal & Bose, 1967; Cox & Potkonjac, 1970). The β-adrenoceptor blocking property of propranolol is thought not likely to contribute to its anti-tremor effect since blockade of β-adrenoceptors by (+)-N-isopropyl-p-nitrophenylethanolamine is ineffective against tremor induced by tremorine in mice (Agarwal & Bose, 1967).

The ability of propranolol to protect significantly against the skeletal myoneural effects of oxotremorine on the rat isolated diaphragm presented here appears to be attributable to a presynaptic action. The mechanism could be diminished transmitter output since propranolol increased the paralytic effect of (+)-tubocurarine and reduced the antagonistic action of TEA on (+)-tubocurarine paralysis. That the effect is presynaptic is also indicated by the failure of propranolol to antagonize responses to acetylcholine of frog rectus abdominis muscle.

In studies of some known anti-Parkinson drugs, Ahmed & Marshall (1962) found a close parallelism between anti-tremorine and anti-acetylcholine effects. That the mechanism by which propranolol inhibits oxotremorine-tremor is unlikely to be due to blockade of peripheral muscarinic receptors is supported by the present experiments using rat blood pressure and guinea-pig ileum.

It is not possible to exclude a central site of action of the anti-tremor effect of propranolol although Hermansen (1968) found that propranolol did not affect oxotremorine-induced analgesia and hypothermia in mice. Cox & Potkonjak (1970) postulated that oxotremorine-tremor was mediated through peripheral propranolol-sensitive receptors perhaps of the same kind as those which on stimulation enhance physiological tremor (Marsden *et al.*, 1967). The present results indicate that such peripheral propranolol-sensitive receptors may be located at the skeletal myoneural junction.

The author wishes to thank Mr S.C. Sharma and Miss Asoka Sengupta for technical assistance. Thanks are also due to Mr D.D. Banerjee and Mr H.N. Dutta for illustrations.

References

AGARWAL, S L & BOSE, D. (1967). A study of the role of brain catecholamines in drug induced tremor. *Br. J. Pharmac.*, **30**, 349–353.

AHMED, A & MARSHALL, P.B. (1962). Relation between antiacetylcholine and antitremorine activity in anti-parkinson and related drugs. *Br. J. Pharmac.*, **18**, 247–254.

AHMED, A. & TAYLOR, N.R.W. (1959). The analysis of drug-induced tremor in mice. *Br. J. Pharmac.*, **14**, 350–354.

BOWMAN, W.C., HEMSWORTH, B A. & RAND, M.J. (1962). Triethylcholine compared with other substances affecting neuromuscular transmission. *Br. J. Pharmac.*, **19**, 198–218.

BÜLBRING, E. (1946). Observations on the isolated phrenic nerve-diaphragm preparation of rat. *Br. J. Pharmac.*, **1**, 38–61.

COLLIER, B. & EXLEY, K.A. (1963). Mechanism of the antagonism by tetraethylammonium of the neuromuscular block due to d-tubocurarine or Ca-deficiency. *Nature, Lond.*, **199**, 702–703.

COX, B & POTKONJAK, D. (1970). Effects of drugs on tremor and increase in brain acetylcholine produced by oxotremorine in rat. *Br. J. Pharmac.*, **38**, 171–180.

CSILLIK, B. (1964). Tremorine, its peripheral action on striated muscle. *Science, N.Y.*, **146**, 765–766.

ELMQVIST, D. & McISSAC, R J. (1967). Oxotremorine at myoneural junction. *Eur. J. Pharmac.*, **1**, 11–14.

GANGULY, D.K. & CHAUDHURI, S.K. (1970). Neuromuscular pharmacology of oxotremorine. *Eur. J. Pharmac.*, **11**, 84–89.

GANGULY, D.K. & SAHA, L. (1972). Pressor response to oxotremorine in atropinised rats. *Experientia*, **28**, 1458–1459.

HERMANSEN, K. (1968). Some pharmacological properties of a new β-adrenergic blocking agent, 1,isopropylamino-3-(O-phenoxyphenoxy)-2-propanol-HCl (ph QA 33). *Acta Pharmac. Tox.*, **26**, 343–362.

JACOBI, H. (1957). Hemmung des Tremors durch β-adrenolytisch wirksame Substanzen. *Naturwissenschaften*, **54**, 94.

MARSDEN, C.D., MEADOWS, J.C., LANGE, G.W. & WATSON, R.S. (1967). Effect of deafferentiation on human physiological tremor. *Lancet*, **2**, 700–702.

OWEN, D.A.L. & MARSDEN, C.D. (1965). Effect of adrenergic β-blockade on Parkinsonian tremor. *Lancet*, **2**, 1259–1262.

SPENCER, P.S.J. (1965). Activity of centrally acting and other drugs against tremor and hypothermia induced in mice by tremorine. *Br. J. Pharmac.*, **25**, 442–455.

(Received December 19, 1974.
Revised July 8, 1975)

Br. J. Pharmac. (1976), **56**, 25–27

PRESSOR EFFECTS OF TRYPTAMINE ANALOGUES

T.R. BOSIN, E. JANE HIXSON & R.P. MAICKEL
Department of Pharmacology, Medical Sciences Program, Indiana University,
Bloomington, Indiana 47401, U.S.A.

1 Methylation of tryptamine in the 1-position had little effect on the potency of the drug as a pressor agent in the intact anaesthetized rat.

2 In contrast, substitution of a benzo[b]thiophene ring system for the indole ring decreased the pressor activity.

3 Pretreatment of the animals with reserpine reduced the pressor effect of tryptamine and its benzo[b]thiophene analogue while increasing the effect of the 1-methylindole analogue.

4 Pretreatment with phenoxybenzamine reduced the pressor effect of all three compounds.

Introduction

Since the effects of tryptamine on guinea-pig isolated ileum were not inhibited by atropine or antihistamines, and, since a strip desensitized by tryptamine was insensitive to 5-hydroxytryptamine and vice versa, Gaddum (1953) concluded there must be a specific tryptamine receptor. Rocha e Silva, Valle & Picarelli (1953) defined a 5-hydroxytryptamine receptor in guinea-pig ileum differing from those for acetylcholine, bradykinin, histamine, nicotine and pilocarpine. Gaddum & Hameed (1954) reported similar receptors in rat uterus and in the vasculature of the rabbit isolated ear. Gaddum & Picarelli (1957) defined two types of tryptamine receptor in the guinea-pig ileum, one blocked by morphine and the other by phenoxybenzamine.

The effect of tryptamine on blood pressure was first noted by Laidlaw (1912). The effects of tryptamine as a pressor substance have been compared to those of 5-hydroxytryptamine by Reid (1951), Reid & Rand (1951) and Page (1952) and ourselves (Hixson, Bosin, Zabik & Maickel, 1973). More recently, Winter, Gessner & Godse (1967) examined the effects of a series of amines on the rat isolated fundus preparation, and found that tryptamine and its benzo[b]thiophene analogue had higher relative intrinsic activities but lower affinities than 5-hydroxytryptamine.

Methods

Adult, male Sprague-Dawley rats (300–350 g) were obtained from Murphy Breeding Laboratories, Plainfield, Indiana, U.S.A. and maintained on Purina Lab Chow and tap water *ad lib* for 7–10 days before experimental use. Pentobarbitone sodium (Nembutal), reserpine (Serpasil), heparin, and tryptamine hydrochloride were purchased from commercial sources. Phenoxybenzamine hydrochloride (Dibenzyline) was kindly supplied by Smith-Kline and French Laboratories. 3-(2-Aminoethyl)benzo[b]thiophene (tryptamine-S), and 1-methyl-3-(2-aminoethyl)-indole (tryptamine-1-Me) were synthesized and supplied by Dr E. Campaigne, Department of Chemistry, Indiana University.

Surgical procedure

Animals were anaesthetized with pentobarbitone, intraperitoneally as follows: controls—60 mg/kg; reserpine pretreated—50 mg/kg; phenoxybenzamine pretreated—45 mg/kg. When pinching the tail elicited no response, the rats were placed on their backs on a small animal operating board (Interex Corp.). The trachea was cannulated to insure a free airway and the common carotid artery was ligated at the superior end then clamped at the inferior end. A cannula of PE 50 tubing was inserted into the artery through a small incision, attached to a 23 gauge needle, and connected via a three-way stopcock to a Statham strain gauge model P23AA and to the pressure bottle of a mercury manometer system. The system was filled with 0.93% w/v NaCl solution containing heparin 1000 u/ml. The strain gauge was connected to the coupler of an Offner Type RB Dynograph (Beckman Instrument, Inc.), or, via a Bridge Preamplifier (Narco Biosystems, Inc.) to a Physiograph model PMP-4A (Narco Biosystems, Inc.). The system was flushed briefly with the heparin/saline solution, and blood pressure recorded to confirm the intact system.

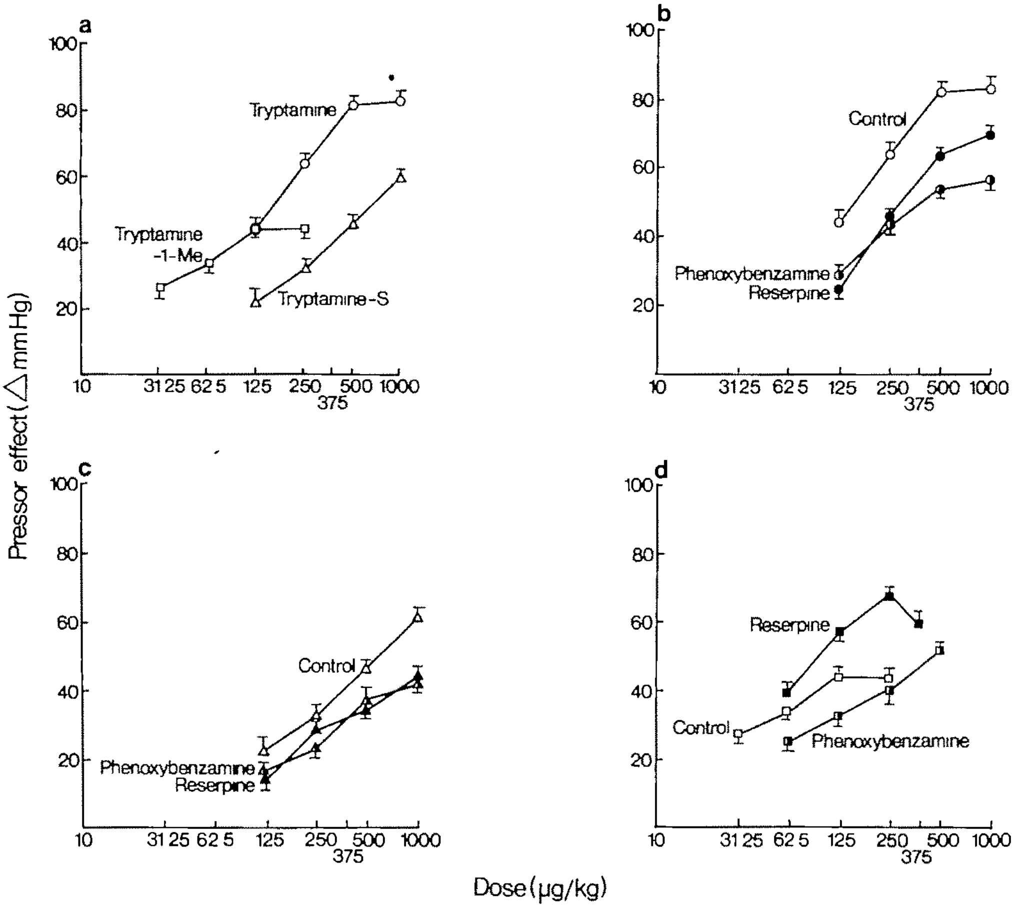


Figure 1 Pressor effects of tryptamine and analogues. Baseline blood pressure values were 110 ± 5 mmHg in control rats, 60 ± 4 mmHg in reserpine-pretreated rats, and 86 ± 5 mmHg in phenoxybenzamine-pretreated rats (a) Effects of tryptamine and analogues in control rats; (b) effects of pretreatments on pressor responses to tryptamine; (c) tryptamine-S, and (d) tryptamine-1-Me Vertical bars indicate s.e. mean.

The external jugular vein was then exposed for a distance of 1 to 1.5 cm. A cannula of PE 50 tubing was inserted, attached to a 23 gauge needle and connected via a three-way stopcock to a 3 ml syringe containing a flushing solution of the heparin/saline solution used above, and a 1 ml syringe containing the test drug.

Testing method

Test drugs were made up in the heparin/saline solution such that a volume of 0.1 ml contained a specific dose of the drug. Each of four animals was given four doses of drug four times, in a Latin square design, over a period of 1–2 hours. In addition, a Latin square was designed based on the sequence of the four cycles, so that each animal received a different sequence of cycles. Reserpine (10 mg/kg i.p.) was given 22–26 h before testing. Phenoxybenzamine (5 mg/kg i.p.) was given 1.5 h before testing.

Results

In control animals tryptamine caused a significant pressor response over a range of 125–1000 µg/kg. Both of the analogues also showed a pressor effect; tryptamine-S was less potent than tryptamine, while tryptamine-1-Me had a similar potency to tryptamine (Figure 1a). Transitory respiratory paralysis was seen occasionally after 500 µg/kg and usually after 1000 µg/kg of tryptamine and tryptamine-S. Tryptamine-1-Me was more toxic; 1000 and

500 µg/kg invariably caused a fatal cessation of respiration.

Pretreatment of rats with a single dose of reserpine reduced the pressor effects of typtamine and trypamine-S significantly (Figures 1b, c). Respiratory paralysis of brief duration occurred after the higher doses with both tryptamine and tryptamine-S. In contrast, the pressor responses to the doses of tryptamine-1-Me given to reserpine-treated animals, 62.5, 125 and 250 µg/kg, were greater in the same doses than in normal animals (Figure 1c).

In animals pretreated with phenoxybenzamine, the pressor effects of all three compounds were significantly reduced when compared to their actions in control animals (Figures 1b, c, d). Of particular interest is the fact that respiratory paralysis due to the pressor agents was a rare occurrence after phenoxybenzamine pretreatment.

Discussion

The scientific literature contains a variety of references to the existence of tryptamine receptors (Erspamer, 1954). However, it is difficult to extrapolate from responses of isolated preparations to those of the vasculature in the intact animal. While tryptamine may interact with its own specific peripheral receptor(s) to increase blood pressure, one cannot rule out the possibility of a centrally-mediated component.

Since the dose-response curves were not parallel the relative potencies of the analogues depended to some extent on the dose. Tryptamine-S was less potent than the other two compounds. Both pretreatments (reserpine and phenoxybenzamine) reduced the pressor effects of tryptamine and tryptamine-S. In contrast, reserpine pretreatment increased the pressor response to tryptamine-1-Me, while phenoxybenzamine caused a decrease in the pressor activity of this analogue. These results argue against the three agonists acting on a single receptor.

Chiu, Harrison, Maickel & Bosin (1973) have reported that the tryptamine analogues have lipid solubilities in the order: tryptamine-S > tryptamine-1-Me > tryptamine; the pK_a values and percent ionized at pH 7.4 for the three agonists are virtually identical. Thus, the differences in pharmacological activity described in this paper cannot be due merely to physicochemical differences; therefore, the differences in potency must be due to differences in the mechanism(s) by which these agonists exert their effect on blood pressure. It has been shown that tryptamine does cross the blood-brain barrier (Erspamer, 1961). The physicochemical characteristics of the two analogues indicate that they should also cross the barrier. Thus, a central component may be involved. From the data presented here, it is impossible to speculate as to what type of receptor or combination of receptors is involved.

This work was supported in part by USPHS grants MH-18852, NS-09672 and K02-MH-41093 and by NASA grant NGL-15-003-117. It was taken in part from a thesis submitted by E.J. Hixson in partial fulfillment of the requirements for the M.S. degree in Pharmacology, Indiana University, 1973.

References

CHIU, P., HARRISON, S.D., JR., MAICKEL, R.P. & BOSIN, T.R. (1973). Chemical pharmacology of benzo[b]-thiophene derivatives. XI. Physicochemical properties of benzo[b]thiophene and 1-methylindole analogs of indolic compounds of pharmacological interest. *The Pharmacologist*, **15**, 184.

ERSPAMER, V. (1954). Pharmacology of indolealkylamines. *Pharmacol. Rev.*, **6**, 425–487.

ERSPAMER, V. (1961). Recent research in the field of 5HT and related indolealkylamines. *Prog. Drug Res.*, **3**, 151–367.

GADDUM, J.H. (1953). Tryptamine receptors. *J. Physiol.*, **119**, 363–368.

GADDUM, J.H. & HAMEED, K.A. (1954). Drugs which antagonize 5-hydroxytryptamine. *Br. J. Pharmac. Chemother.*, **9**, 240–248.

GADDUM, J.H. & PICARELLI, Z.P. (1957). Two kinds of tryptamine receptors. *Br. J. Pharmac. Chemother.*, **12**, 323–328.

HIXSON, E.J., BOSIN, T.R., ZABIK, J.E. & MAICKEL, R.P. (1973). Effects of benzo[b]thiophene and 1-methylindole analogs of indolealkylamines on rat blood pressure. *The Pharmacologist*, **15**, 180.

LAIDLAW, P.P. (1912). The physiological action of indolethylamine. *Biochem. J.*, **6**, 141–150.

PAGE, I.H. (1952). The vascular action of natural serotonin, 5- and 7-hydroxytryptamine and tryptamine. *J. Pharmac. exp. Ther.*, **105**, 58–73.

REID, G. (1951). The pharmacology of tryptamine. *Aust. J. exp. Biol. Med. Sci.*, **29**, 101–116.

REID, G. & RAND, M. (1951). Physiological actions of the partially purified serum vasoconstrictor (serotonin). *Aust. J. exp. Biol. Med. Sci.*, **29**, 401–415.

ROCHA E SILVA, M., VALLE, J.R & PICARELLI, Z.P. (1953). A pharmacological analysis of the mode of action of serotonin (5-hydroxytryptamine) upon the guinea pig ileum. *Br. J. Pharmac. Chemother.*, **8**, 378–388.

WINTER, J.C., GESSNER, P.K. & GODSE, D.D. (1967). Synthesis of some 3-indenealkylamines. Comparison of the biological activity of 3-indenealkylamines and 3-benzo[b]thiophenealkylamines with their tryptamine isosteres. *J. med. Chem.*, **10**, 856–859

(Received February 11, 1975.
Revised April 7, 1975)

Br. J. Pharmac. (1976), **56**, 29–38

EFFECTS OF 5,7-DIHYDROXYTRYPTAMINE ON AN IDENTIFIED 5-HYDROXYTRYPTAMINE-CONTAINING NEURONE IN THE CENTRAL NERVOUS SYSTEM OF THE SNAIL *Helix pomatia*

N.N. OSBORNE

Max-Planck-Institut fur experimentelle Medizin, Forschungsstelle Neurochemie,
3400 Gottingen, Germany

V.W. PENTREATH

Wellcome Laboratories of Pharmacology, Gatty Marine Laboratory,
University of St Andrews, St. Andrews, Fife, Scotland

1 The effect of 5,7-dihydroxytryptamine (5,7-DHT) on an identified 5-hydroxytryptamine (5-HT)-containing neurone in the CNS of the snail was studied by histochemical, biochemical and electrophysiological methods.

2 Low concentrations of 5,7-DHT decreased the endogenous 5-HT content of the neurone without affecting the amino acids, while relatively large amounts of the drug proportionately lowered 5-HT and in addition slightly decreased the tryptophan and methionine content of the cell.

3 5,7-DHT blocked the uptake of $[^3H]$-5-HT into the neurone; the close analogue 5,6-DHT was more potent.

4 As well as slightly influencing the accumulation of $[^3H]$-tryptophan by the neurone 5,7-DHT inhibited the metabolism of this amino acid to form 5-HT, probably by affecting the enzyme tryptophan-hydroxylase.

5 5,7-DHT produced a postsynaptic blockade of transmission from the neurone by blocking the 5-HT receptors of the follower neurones. This effect appeared to be specific for 5-HT receptors.

6 The data support the idea that 5,7-DHT is neurotoxic for indoleamine-containing neurones.

Introduction

In each cerebral ganglion of the snail *Helix pomatia*, and also a number of other gastropods, there is a large or giant neurone located in the anterior-ventral position (Kandel & Tauc, 1966; Cottrell & Osborne, 1970; Osborne & Cottrell, 1971). Conclusive evidence has shown that these giant neurones contain 5-hydroxytryptamine (5-HT or serotonin) (Osborne, 1971; Osborne & Cottrell, 1971, 1972; Osborne, 1973b) and the cells have consequently been termed 'giant serotonin cells' or 'GSCs'. The membrane properties of the GSGs in *Helix* have been studied by Kandel & Tauc (1966), who also showed each GSC to send an axon to the ipsilateral exterior lip nerve and one to each cerebral buccal connection. Further electrophysiological and autoradiographic studies on these cells (Cottrell, 1970, 1971; Pentreath & Cottrell, 1972, 1974; Cottrell & Macon, 1974) demonstrated that the two GSCs form synaptic excitatory links with other giant neurones in the buccal ganglia. Unlike the GSCs, the buccal neurones lack 5-HT or catecholamines, and only the GSCs contain the enzymes for synthesizing 5-HT from tryptophan (Osborne, 1973c) or 5-hydroxytryptophan (Cottrell & Powell, 1971; Osborne, 1972).

The experiments reported here describe the effects of 5,7-dihydroxytryptamine (5,7-DHT) on the GSCs studied by histochemical and biochemical procedures, and on synaptic transmission from the GSCs. In the vertebrates, 5,7-DHT has a neurotoxic mode of action on 5-HT neurones, and to a lesser extent on noradrenaline neurones, but has apparently no effect on dopamine neurones in the CNS (Baumgarten, Björklund, Lachenmayer & Nobin, 1973). The drug is also known to influence 5-HT metabolism in brain tissue of the snail (Osborne, 1974a). The present experiments were also designed to compare the effects of 5,7-DHT on the GSC with those known to occur on 5-HT-containing neuronal systems in the vertebrates.

Methods

Specimens of *Helix pomatia* were obtained from Gerard and Haig Ltd., East Preston, Sussex, or from Alfred Koch, 345 Holzminden, Germany and were kept at room temperature (18–22°C) in aquaria which contained a little water. All experiments were performed at room temperature.

Fluorescence microscopy

Snail nervous tissue was superfused at a constant rate with snail's saline (Meng, 1960) containing 10^{-3} M ascorbic acid and various concentrations of 5,7-DHT (10^{-6} to 5×10^{-3} M) for 15, 30, 90 or 150 minutes. Ascorbic acid was used to prevent the breakdown of 5,7-DHT. Control preparations were superfused for the same period of time in snail's saline containing ascorbic acid only. Thereafter the supraoesophageal ganglia were dissected, frozen in liquid N_2 and freeze-dried for at least two days at –35°C in a vacuum of 10^{-3} torr in an Edwards-Pearse Tissue Dryer, model EPD3. Dried tissue was exposed to formaldehyde gas for 1 h at 80°C (Falck & Owman, 1965). The vapour was obtained from paraformaldehyde (Merck) which had been kept at a relative humidity of 70% (Hamberger, Malmfors & Sachs, 1965). The nervous tissue was subsequently embedded under reduced pressure in paraffin wax and 10 μm sections were examined with a Leitz microscope fitted with a darkfield condenser, appropriate filters and a mercury vapour lamp (Corrodi & Johnson, 1967).

Amino acid and 5-hydroxytryptamine content experiments

Snails were injected through their foot musculature once a day over a period of 4 days with 0.1 ml snail's saline containing 10^{-3} M ascorbic acid and various concentrations of 5,7-DHT so that individual animals received between 10^{-5} and 10^{-3} M of the drug. Individual GSCs were then dissected from the snails as described elsewhere (Osborne, 1974b), and placed in a micro-tube containing 15 μl 50% acetone in water. After collecting 4–6 GSCs from animals injected with the same amount of 5,7-DHT, the samples were frozen in liquid nitrogen and then thawed. This 'shocking' process was repeated three times before centrifuging the tubes at 1500 ***g*** for 60 minutes. Thereafter the supernatant of each sample was analysed either fluorimetrically by the procedure of McCaman, Weinreich & McCaman (1973) for their 5-HT content, or by a micro-dansylation chromatography technique (see reviews Neuhoff, 1973; Osborne, 1973a) for their amino acid content.

[^{3}H]-Tryptophan metabolism experiments

After pinning the dissected supraoesophageal ganglia on to the plastic base of a small chamber (vol. 1 ml) which contained snail's saline, the connective tissue surrounding the GSCs was dissected with fine forceps. The saline was then replaced by a fresh solution containing 10^{-3} M ascorbic acid and [^{3}H]-tryptophan (Radiochemical Centre, Amersham, England, specific activity 5.58 Ci/mmol; 75 μCi/ml) together with 10^{-4} M of 5,7-DHT. In controls 5,7-DHT was omitted. After an incubation time of between 30 and 150 min, the physiological solution containing the radioactivity was removed and the tissue rinsed with physiological saline for 10 minutes. The GSCs were then dissected and transferred by means of a fine pipette (see Osborne, 1974b) to microtubes containing 15 μl 50% actetone in 0.01 N HCl. After collecting 10 cells from nervous tissues treated in the same way, the sample was frozen in liquid N_2 and thawed. This 'shocking' process was repeated three times before centrifuging the tube at 1500 ***g*** for 60 minutes. Thereafter the supernatant was applied to a 5 x 5 cm silica gel precoated plate (Merck –60), together with small amounts (approx. 2 μg of each substance) of carrier tryptophan, 5-hydroxytryptophan (5-HTP), 5-HT and 5-hydroxyindoleacetic acid (5-HIAA). Chromatograms were then developed by ascending chromatography in covered beakers using *n*-butanol/pyridine/acetic acid/water (15:2:3:5 by vol.). Substances were subsequently identified by first spraying the chromatograms with 1% potassium ferricyanide in ammonium hydroxide solution followed by ninhydrin. 5-HTP, 5-HT and 5-HIAA appeared brown in colour and tryptophan blue. Individual substances were then eluted from the silica gel with methanol, the extraction was repeated five times, and the combined eluates from a single substance were dried in counting vials with a stream of N_2. Scintillation fluid (4 g PPO and 0.1 g POPOP/litre toluene) was added to the vials and the radioactivity counted in a Packard scintillation spectrometer.

The residual protein precipitates of the cells were dissolved in 10–20 μl of 1 N NaOH and the protein estimated by the microprocedure of Lowry, Rosebrough, Farr & Randall (1951). A series of protein standards, 0.5 μg to 6 μg of serum albumin, was carried through the procedure and used as reference standards.

Uptake experiments

GSCs (14–18) were rapidly dissected from a number of snails and preincubated for 15 min in 2 ml snail's saline at 25°C. [^{3}H]-5-HT (Radiochemical Centre, Amersham, England, specific activity 17.3 Ci/mmol) at a concentration of 10^{-5} M, containing 10^{-3} M ascorbic acid either alone or together with 5×10^{-5} M 5,7-DHT, or other substances were added and the incubation allowed to continue for 30 minutes. [^{3}H]-5-HT is known to be taken up by snail nervous tissue by a high affinity process under these conditions (Osborne, Hiripi & Neuhoff, 1975). Incubations were terminated by rapid filtration of the sample through filters (0.8 µm pore size) followed by a rapid wash with 2 ml of fresh snail's saline in order to remove all traces of non-occluded radioactivity. After drying by leaving overnight at room temperature, filters were placed face up in scintillation vials and after the addition of 10 ml toluene scintillation fluid, their ^{3}H- content was measured by scintillation counting. The efficiency of the counting was estimated by including standards with each batch of analysis. These standards consisted of filters through which non-radioactive incubated tissue samples had been filtered, and to which were then added known amounts of [^{3}H]-5-HT or [^{3}H]-toluene. The efficiency of counting varied between 18 and 25 per cent.

Electrophysiological experiments

The isolated cerebral and buccal ganglia were pinned to the base of a 5 ml acrylic chamber. The preparation was bathed with continuously flowing physiological solution, which was stopped when the effects of drugs were being tested. The cerebral ganglia were pinned ventral surface uppermost to expose the GSC. Most of the connective tissue layer above the GSC and the buccal ganglia neurones to be impaled was removed by dissection with fine-tipped forceps. The GSC and the postsynaptic buccal neurone being studied were each impaled with a double-barrelled electrode. Both barrels were filled with 0.6 M K_2SO_4; one barrel was used for recording, the other for stimulating.

In some experiments the cerebral buccal connection was stimulated with a plastic suction electrode connected in series with a stimulus isolation unit. Records were made on a Brush 220 series two-channel link recorder. Conventional stimulating and amplifying equipment was used.

Two types of experiments were carried out with 5,7-DHT. In the first, snails were injected each morning over a period of 3 days with 5,7-DHT (0.5 ml of snail's saline containing 2 µg/ml and 10^{-3} M ascorbic acid). The cerebral and buccal ganglia were then dissected and an electrophysiological analysis was made of the effect of the drug on the synaptic connections between the GSCs and the buccal neurones. Control animals were injected with the same volumes of saline containing only 10^{-3} M ascorbic acid. In other experiments the effect of 5,7-DHT was tested on isolated cerebral and buccal ganglia. 5,7-DHT was added to the organ bath to give a concentration of 2×10^{-4} M to 1×10^{-3} M. The nervous tissues were exposed to the drug for 10 s to 3 hours. In some studies KCl was omitted from the saline solution. 5-HT and acetylcholine (ACh) were applied to the postsynaptic neurones in the buccal ganglia from a small bore (150 µm tip diameter) syringe. Successive 5-HT applications were separated by intervals of at least 15 min because of receptor desensitization. The concentration of 5-HT was 2×10^{-5} M, that of ACh was 3×10^{-6} M.

Results

Fluorescence microscopy

Control preparations showed the GSC with its yellow fluorescing cytoplasm (indicative of 5-HT) to be situated on the ventral surface of the metacerebral part of each cerebral ganglion. Characteristically, a group of small (10–20 µm), intensely fluorescing, green cells (indicative of dopamine) was seen around the medial edge of each of the GSCs (Figure 1). As with other fluorescent cells, the nucleus of the GSC did not fluoresce. The fluorescence associated with the cytoplasm of the control GSCs was slightly mottled in appearance and not evenly distributed, as would normally be the case with the GSCs from freshly dissected ganglia (see Osborne & Cottrell, 1971). Following exposure of ganglia to 5,7-DHT, a number of changes occurred in the amine fluorescence. The fluorescence in the GSCs was slightly increased by all concentrations of 5,7-DHT perfused over a period of 15 minutes. However, over a perfusion period of 30–90 min the yellow fluorescence associated with the GSC was markedly decreased by either 10^{-6} or 5×10^{-3}M 5,7-DHT (Figure 1). Little alteration in the nature of the fluorescence of the green cells was noticed (Figure 1). Over a longer period of perfusion, viz. 150 min, 10^{-6} and 10^{-5} M 5,7-DHT produced effects similar to those observed for a perfusion period of 90 minutes. In contrast, 10^{-4} and 5×10^{-3} M 5,7-DHT (150 min perfusion) not only caused the fluorescence in the GSCs to fade, but also made the cell appear slightly swollen and apparently damaged. Moreover, a decrease in the fluorescence of the green cells and neuropile region was noted.

Effect of 5,7-dihydroxytryptamine on the amino acid and 5-hydroxytryptamine content of the GSC

The amino acids and related compounds which occur in the GSC and which react with [^{14}C]-dansyl chloride have been described elsewhere (see Osborne & Cottrell, 1972; Osborne, 1973a). No significant change in the content of any substance in the GSC was produced by pretreatment of snails with 5,7-DHT at either 10^{-5} M or 10^{-4} M. Higher concentrations of the drug (10^{-3} M) produced a slight but significant decrease in the content of tryptophan and methionine.

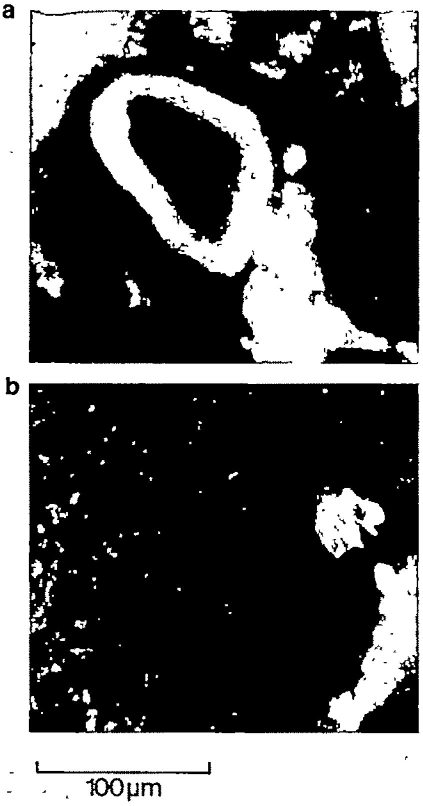


Figure 1 Sections through the cerebral ganglia of *Helix pomatia* showing the appearance of a 'giant serotonin cell' (GSC) from an untreated animal (a) and from a snail treated with 5,7-dihydroxytryptamine (5,7-DHT) (b). The tissue was processed by the formaldehyde procedure for localizing monoamines, the normal GSCs appearing yellow in colour (showing them to contain 5-hydroxytryptamine) and in close association with small green fluorescing cells (indicating the presence of dopamine). The yellow fluorescence of the GSC is decreased by 5,7-DHT.

In our hands the sensitivity of the method described by McCaman *et al.* (1973) was of the order of 500 pg 5-HT. From a combination of 10 independent determinations (each determination required between 4 and 6 GSCs), each GSC was estimated to contain 700 ± 50 pg 5-HT. This agrees with our previous findings where other procedures were used to analyse the 5-HT content of the GSCs (Cottrell & Osborne, 1970; Osborne, 1973a). Figure 2 shows 5,7-DHT (1×10^{-5} M) to cause almost a 50% drop in the 5-HT content when injected into snails. With increasing doses of 5,7-DHT, the 5-HT content was lowered to an even greater extent. The highest dose used (10^{-3} M) produced an 85% decrease of 5-HT in the GSC.

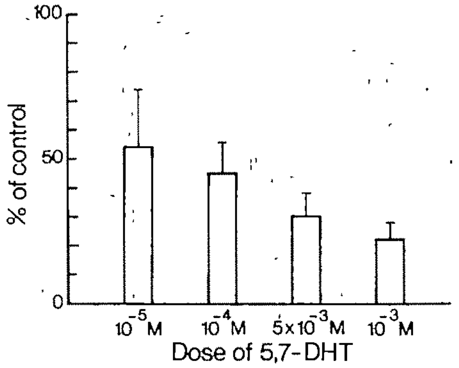


Figure 2 Effect of four different doses of 5,7-dihydroxytryptamine (5,7-DHT) on 'giant serotonin cell' 5-hydroxytryptamine (5-HT) content. The columns give means of four determinations. Vertical lines show s.e. mean. 100% 5-HT = 700 ± 50 pg.

Effect of 5,7-dihydroxytryptamine on tryptophan metabolism in the GSC

As can be seen from Figure 3, the chromatographic procedure used allows a separation of 5-HT and metabolites on plates measuring 5 x 5 cm. Though the procedure was sensitive, allowing less than 1 µg of substance to be detected, it was not sensitive enough to measure the endogenous content of 5-HT and metabolites in as many as 18 GSCs. Examination of chromatograms of extracts from the GSCs which had been exposed to [^{3}H]-tryptophan showed radioactivity to be associated with three substances only: tryptophan, 5-hydroxytryptophan (5-HTP) and 5-HT. This confirmed previous data (Osborne, 1973c). From Table 1 it can be seen that only very small amounts of the accumulated [^{3}H]-tryptophan are actually metabolized and that the quantity of [^{3}H]-5-HT formed increases with time while the [^{3}H]-5-HTP content stabilizes after 60 minutes. The effect of 10^{-4}M 5,7-DHT on the accumulation and metabolism of [^{3}H]-tryptophan is also shown in Table 1. The accumulation of [^{3}H]-tryptophan is initially unaffected by the drug but over a longer period of time a definite inhibition occurs. Moreover, the drug drastically affects the metabolism of the accumulated [^{3}H]-tryptophan, in particular the amount of [^{3}H]-5-HT formed. In contrast the content of [^{3}H]-5-HTP formed from the [^{3}H]-tryptophan taken up is not significantly affected by 5,7-DHT.

Effect of 5,7-dihydroxytryptamine on the uptake of 5-hydroxytryptamine by the GSC

Previous studies have shown that [^{3}H]-5-HT uptake in the snail brain is inhibited by 5,7-DHT with an ID_{50} of

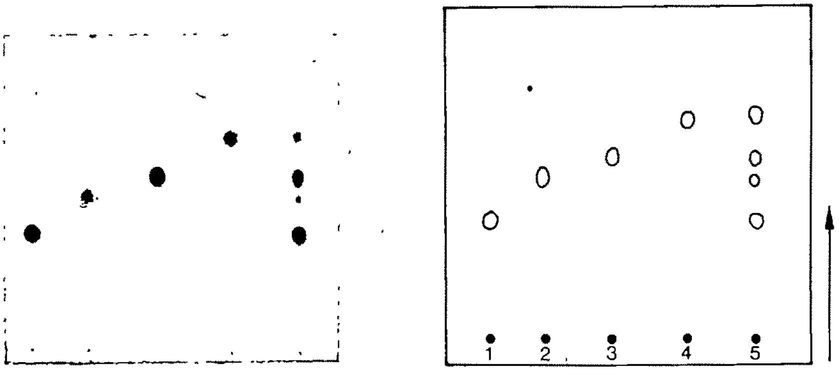


Figure 3 Chromatogram (5 × 5 cm) and map to show the separation of 5-hydroxytryptamine (5-HT) and metabolites following one dimensional chromatography. The direction of chromatography is indicated by the arrow and solution used was *n*-butanol/pyridine/acetic acid/water (15.2.3.:5) 1 = 5-hydroxytryptophan, 2 = tryptophan, 3 = 5-HT, 4 = 5-hydroxyindoleacetic acid, 5 = mixture.

10^{-4} M (see Osborne *et al.*, 1975). It was therefore decided that in order to compare the relative potency of 5,7-DHT and other indole derivatives towards the uptake of [^{3}H]-5-HT by the GSCs, slightly smaller concentrations of substances (5×10^{-5} M) would be tested. Figure 4 shows that at this concentration 5,7-DHT inhibited the accumulation of [^{3}H]-5-HT by 56%. In contrast, the indole derivatives 5-hydroxyindole (5-HI), 5-HTP and 5-hydroxyindoleacetic acid (5-HIAA) only inhibited the uptake by between 7 and 15%, while 5,6-DHT was the most potent, reducing the accumulation of radioactive amine by 62%.

Electrophysiology

Stimulation of the GSC to produce a burst of action potentials resulted in excitatory responses in three large identifiable neurones (anterior, middle and posterior) on the lateral borders of each buccal ganglion (Cottrell, 1970, 1971; Cottrell & Macon, 1974). The input on to the anterior cells was different from that to the middle and posterior cells and was characterized by its long-lasting nature (Figure 5b). When the middle buccal neurone was artificially hyperpolarized, the excitatory response could be seen

Table 1 The effect of 5,7-dihydroxytryptamine (5,7-DHT) on the incorporation of radioactivity from [^{3}H]-tryptophan into 5-hydroxytryptophan (5-HTP) and 5-hydroxytryptamine (5-HT) in 'giant serotonin cells' (GSCs).

Time (min)	*Condition*	*Tryptophan*	*5-HTP*	*5-HT*
30	Saline alone	14,000 ± 200	641 ± 34	582 ± 34
	Saline + 5,7-DHT	13,942 ± 240	632 ± 38	641 ± 32
45	Saline alone	26,000 ± 240	689 ± 38	640 ± 40
	Saline + 5,7-DHT	25,210 ± 230	700 ± 37	630 ± 33
60	Saline alone	38,120 ± 439	754 ± 39	820 ± 48
	Saline + 5,7-DHT	32,000 ± 384	714 ± 36	742 ± 68
90	Saline alone	39,800 ± 880	810 ± 40	1000 ± 68
	Saline + 5,7-DHT	34,840 ± 600	765 ± 38	800 ± 44*
120	Saline alone	47,700 ± 600	820 ± 45	1840 ± 54
	Saline + 5,7-DHT	39,120 ± 594*	700 ± 50	1000 ± 50*
150	Saline alone	50,200 ± 398	829 ± 39	1840 ± 60
	Saline + 5,7-DHT	42,400 ± 520*	681 ± 48	900 ± 39*

Incorporation of radioactivity in d/min per mg protein was measured after incubation of tissue in [^{3}H]-tryptophan for varying periods of time. Results are mean values ± s.e. mean of 4 experiments.
* $P < 0.05$ and therefore significantly different by Student's *t* test

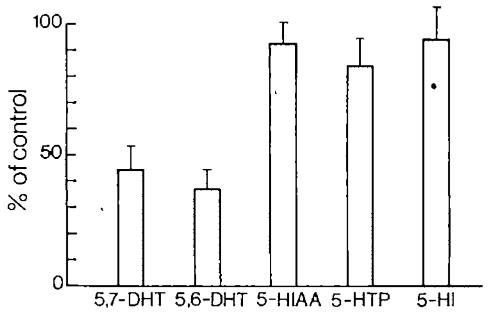


Figure 4 Effect of accumulation of [^{3}H]-5-hydroxytryptamine (5-HT) (10^{-8} M) by 'giant serotonin cells' in the presence of various indole derivatives (5×10^{-5}M) at room temperature (24°C) Each value is the mean of four experiments Vertical lines show s e mean

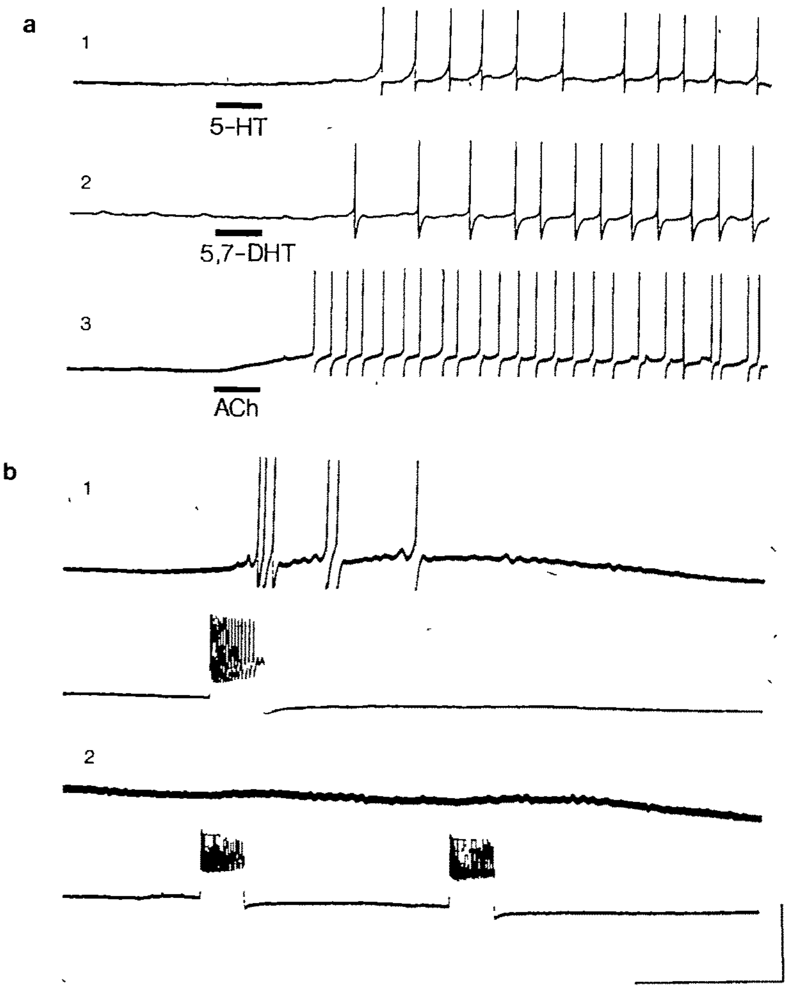


Figure 5 Effect of drugs on the middle buccal cell (a), and of 5,7-dihydroxytryptamine (5,7-DHT) on the response to 'giant serotonin cell' (GSC) stimulation of the anterior buccal cell (b) Records (a1) and (a2) are from the same preparation, (a3) and (b) are from different preparations. (a1–3) show the effect of applying 5-

to be composed of individual e.p.s.ps whose amplitude was increased at greater levels of hyperpolarization (Figure 6a). The buccal neurones were depolarized by low concentrations of 5-HT (Figure 5a).

No marked change in response to GSC stimulation was observed in either the middle or anterior cells in snails which had been injected over three days with 5,7-DHT. This finding was surprising because the drug decreased the level of 5-HT within the GSC soma. However, it is possible that any small change in e.p.s.p. size would only be apparent after analysing large numbers of drug treated animals. Such extensive analysis was not undertaken in the present study. This procedure would be necessary to rule out the natural variation in e.p.s.p. size between different animals. Cottrell & Macon (1974) found that reserpine treatment reduced, but did not abolish, the excitatory responses in the middle buccal cell.

5,7-DHT mimicked the effect of 5-HT on the middle and anterior buccal neurones; in concentrations of 2×10^{-4} M to 1×10^{-3} M it caused depolarization and firing of action potentials (Figure 5a). If, after such a depolarization the preparation was briefly washed, it was found that the effect of 5-HT (2×10^{-5} M), but not ACh (5×10^{-6} M), was abolished. This suggested that the drug blocked 5-HT receptors on the somata of these neurones. The responses to 5-HT recovered gradually with washing (about 15 minutes).

The action of 5,7-DHT was tested on the buccal cell responses in freshly isolated preparations. At a concentration of 6×10^{-4} M to 2×10^{-3} M there was a rapid (approximately 2 min) abolition of input from the GSC on to the middle (Figure 6a) and anterior (Figure 5b) cells. There was an accompanying blockade of the depolarizing response to 5-HT whereas response to ACh was unaffected. After washing for approximately 15 min there was a gradual and eventually (approx. 1 h washing) complete recovery of e.p.s.ps arising from the GSC input, and concomitant recovery of the response to 5-HT. After prolonged exposure to 5,7-DHT (1–2 h), recovery of the responses to GSC stimulation and to 5-HT required a longer period of washing (1–3 hours).

During periods in which the action of 5,7-DHT was being removed by washing, an unusual response to GSC stimulation of the middle buccal cells was observed in several preparations. Instead of excitation, a weak inhibition resulted (Figure 6b). The reason for this effect is not clear, although it appeared to be dependent upon a number of factors, such as previous concentration of 5,7-DHT employed, the time after commencement of washing, and frequency of GSC stimulation. This effect was not observed when the electrode was removed from the GSC and current passed into the bath. The effect was not mimicked by the addition of 5-HT (2×10^{-5} M) to the buccal cell soma, and was lost when the cell's sensitivity to 5-HT recovered.

In order to test whether the action of 5,7-DHT was specific and not the result of a reduction in membrane resistance, the effect of the drug on the membrane resistance of several middle buccal cells before and during its application was tested. This was evaluated by measuring the amplitude of potentials produced by square hyperpolarizing current impulses of constant intensity and 1 s duration given through one barrel of the double-barrelled recording electrode. No appreciable (max. 7%) reduction in membrane resistance could be recorded.

Extra evidence for the specificity of the effect of 5,7-DHT on the e.p.s.ps elicited by the GSC was obtained by stimulating the cerebro-buccal connective to produce e.p.s.ps from a different source. The results are shown in Figure 6c. Relatively large e.p.s.ps were recorded in the middle buccal cell which were probably due to the release of ACh. 5,7-DHT at a concentration of 10^{-3} M had little effect on these e.p.s.ps. 5-HT (4×10^{-5} M) also had little effect on these e.p.s.ps, but both drugs caused a similar but slight change in the time-course of individual e.p.s.ps (Figure 6c).

The results show that 5,7-DHT can produce an apparently specific blockade of 5-hydroxytryptaminergic transmission by blocking 5-HT receptors.

Figure 5 (cont.)

hydroxytryptamine (5-HT) (2×10^{-5} M), 5,7-DHT (3×10^{-4} M) and acetylcholine (ACh) (3×10^{-6} M) to the middle buccal cell The drugs were added at time indicated by the bars (b) Shows electrical responses in the ipsilateral anterior buccal cell (upper trace in each record) to GSC stimulation (bottom traces) (b1) Control response consisting of a relatively persistent depolarization. It was not possible to detect individual e.p.s.ps in this neurone with increased levels of hyperpolarization, probably because the cell exhibits anomalous rectification (see Cottrell & Macon, 1974). (b2) Responses 2 min after the application of 6×10^{-4} M 5,7-DHT. The excitation produced by stimulating the GSC is greatly reduced even though the GSC was made to fire at a higher frequency. Time scale 10 s Voltage calibration, (a1–3) (middle buccal cells) 40 mV; (b1,2) top traces (anterior buccal cell) 20 mV, bottom traces (GSC) 80 mV.

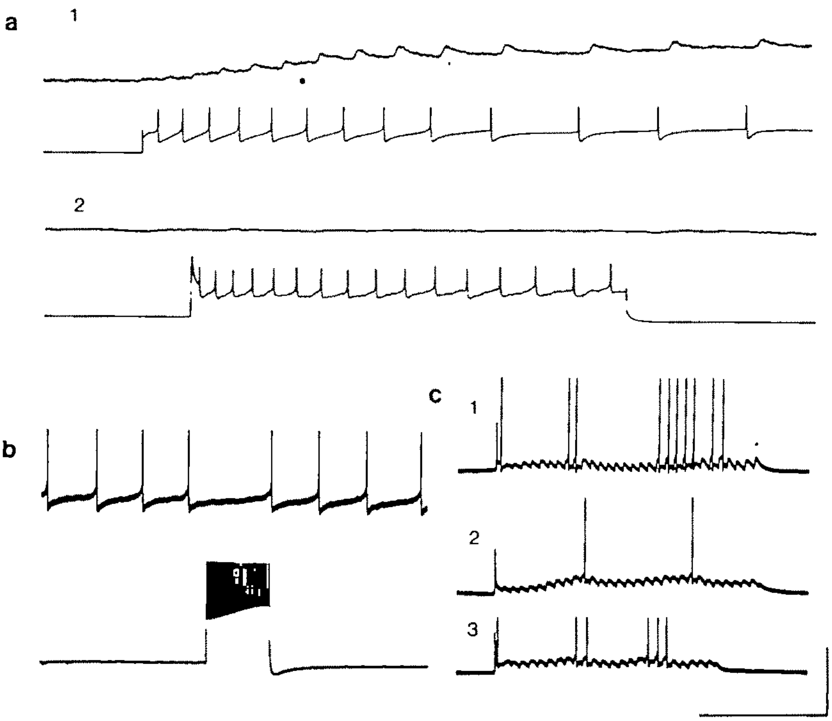


Figure 6 Effect of 5,7-dihydroxytryptamine (5,7-DHT) on the excitatory responses of the ipsilateral middle buccal cell to stimulation of the 'giant serotonin cell' (GSC) (a,b), and on the excitatory responses of the middle buccal cell to stimulation of the ipsilateral cerebro-buccal connective (c). (a) and (b) were obtained from different preparations bathed in KCl-free solution, (c) is a different preparation in normal (Meng's) saline. In (a) the middle cell (top trace in each record) has been artificially hyperpolarized from −55 mV to −100 mV (a1) shows the control e.p s.ps which facilitate and summate during GSC action potential firing (bottom trace). (a2) shows the complete abolition of the response 3 min after the addition of 1×10^{-3} M 5,7-DHT (b) shows the weak inhibitory effect of GSC firing (bottom trace) on the middle buccal cell (at resting potential) 10 min after the start of washing off 6×10^{-4} M 5,7-DHT (20 min exposure) from this preparation. This effect was observed in three different preparations. (c1) Control e.p s ps elicited in the middle buccal neurone by stimulating the cerebro-buccal connective with 1 ms pulses at 0.6 Hz for approximately 20 seconds. Occasional e.p.s.ps elicit action potentials. (c2) and (c3) show the effect of 5,7-DHT (1×10^{-3} M) and 5-hydroxytryptamine (4×10^{-5} M) respectively on similar periods of stimulation The nervous tissue was exposed to each drug for a period of 20 minutes There is no apparent decrease in amplitude of the individual e.p s ps although in each case they possess slightly different time courses. Time scale in (a) is 2 s, in (b) and (c) is 10 seconds Voltage calibrations, (a1,2) top traces (middle buccal cell) 9 mV, bottom traces (GDC) 120 mV; (b) top trace (middle buccal cell) 50 mV, bottom trace (GSC) 40 mV, (c1–3) (middle buccal cell) (c2) mV

Discussion

The present fluorescent histochemical data describing the effect of 5,7-DHT on a specified 5-HT-containing cell (GSC) are similar to those of previous vertebrate studies on indoleamine neurones (Baumgarten *et al.*, 1973) and analogous to the action of 6-hydroxydopamine on a characterized dopamine-containing neurone (Berry, Pentreath, Turner & Cottrell, 1974). The increase in the amine fluorescence associated with the GSC following perfusion of the CNS with 5,7-DHT for 15 min can be interpreted as an initial uptake of 5,7-DHT by the GSC. Over a longer period of perfusion (30, 90 and 150 min) the toxic effect of even small amounts of accumulated 5,7-DHT resulted in a dramatic decrease in the 5-HT fluorescence. Moreover, under the experimental conditions, longer periods of perfusion with high doses of 5,7-DHT also affected the fluorescence associated with the small green dopamine-containing cells. This is slightly surprising in that there is evidence that 5,7-DHT has little influence upon the dopamine content in vertebrate brain tissue (Baumgarten *et al.*, 1973).

From the biochemical data obtained in this study it is clear that 5,7-DHT causes a marked decrease in the 5-HT content of the GSCs in a similar way to that reported for vertebrate tissues (Baumgarten *et al.*, 1973). It was also observed that the levels of a number of amino acids in the GSC including tyrosine, glutamic acid, glycine, aspartic acid, proline, GABA and alanine were unaffected by 5,7-DHT. The results obtained concerning the various suspected transmitter amino acids are further evidence for the specificity of 5,7-DHT in influencing the indoleamine content only. The reason for the very slight decrease in tryptophan and methionine as a result of high doses of 5,7-DHT is not yet clear.

These findings also showed the effect of 5,7-DHT on the synthesis and uptake of 5-HT by the GSC whereby the indoleamine content of the cell is decreased. The fact that 5,7-DHT alters the metabolism of [^{3}H]-tryptophan in the GSC in a similar way to *p*-chlorophenylalanine (Osborne, 1973c; Osborne & Neuhoff, 1974) suggests that the drug exerts its influence upon tryptophan-hydroxylase, as has been shown in vertebrate nervous tissue (Baumgarten, Victor & Lovenberg, 1973). Moreover, these data demonstrate that the drug also affects to a slight extent the accumulation of [^{3}H]-tryptophan by the GSC. This finding, together with the slight decrease in endogenous tryptophan following treatment with high doses of 5,7-DHT, may be of significance in relation to 5-HT turn-over. The observation that 5,7-DHT is slightly less potent than 5,6-DHT but inhibits the uptake of 5-HT into the GSC is also similar to earlier studies on vertebrate nervous tissues (Horn, Baumgarten & Schlossberger, 1973). However, unlike the studies on vertebrate tissues, much higher doses of 5,7-DHT are required to affect the uptake of 5-HT by at least 50%. This substantiates work on whole brain tissue of the snail where the ID_{50} is even higher for 5,7-DHT (Osborne *et al.*, 1975). However, it is clear that compared to other indole derivatives tested (with the exception of 5,6-DHT), 5,7-DHT is a potent inhibitor of the uptake of 5-HT in the GSC.

The postsynaptic action of the 5,7-DHT on the GSC mediated e.p.s.ps was unexpected. The drug (concentration 10^{-4} to 10^{-3} M) rapidly abolished these e.p.s.ps and also eliminated the depolarizing response to 5-HT. On the other hand, the depolarizing response to applied ACh and to presumed cholinergic e.p.s.ps resulting from stimulation of a different input were not antagonized. Since there was a gradual and parallel recovery of the response to 5-HT and to GSC-mediated responses with washing, the blocking action of 5,7-DHT appears to be relatively specific for 5-HT receptors. In this respect the action of 5,7-DHT on 5-HT receptors is analogous to that discovered for 6-hydroxydopamine on dopamine receptors in the pond snail *Planorbis corneus* (Berry & Cottrell, 1973; Berry *et al.*, 1974).

The experiments described in this study have shown that 5,7-DHT has a number of effects on an indoleamine-containing neurone and thus support the idea that the substance is neurotoxic. As well as altering the uptake and metabolism of 5-HT, 5,7-DHT also inhibits 5-HT transmission by blocking the receptors. However, unlike studies on vertebrate tissues (Baumgarten, Groth, Göthert & Manian, 1974), initial electron microscopical findings on the GSC could not demonstrate any pathological alterations following treatment with 5,7-DHT (Osborne & Pentreath, unpublished data).

We thank Professor V. Neuhoff and Dr G.A. Cottrell for reading the manuscript.

References

BAUMGARTEN, H.G., BJÖRKLUND, A., LACHENMAYER, L.L. & NOBIN, A. (1973). Evaluation of the effects of 5,7-dihydroxytryptamine on serotonin and catecholamine neurons in the rat CNS. *Acta physiol. scand. Suppl.* **391**.

BAUMGARTEN, H.C., GROTH, H.P., GÖTHERT, M. & MANIAN, A.A (1974). The effect of 5,7-dihydroxytryptamine on peripheral adrenergic nerves in the mouse. *Nauyn-Schmiedebergs Arch. Pharmac.*, **282**, 245–254.

BAUMGARTEN, H.C., VICTOR, S.J. & LOVENBERG, W. (1973). Effect of intraventricular injection of 5,7-dihydroxytryptamine on regional tryptophan hydroxylase of rat brain. *J. Neurochem.*, **21**, 251–253.

BERRY, M.S & COTTRELL, G.A. (1973). Post-synaptic blockade of dopaminergic transmission by 6-hydroxydopamine. *J. Pharm. Pharmac.*, **25**, 1010.

BERRY, M.S., PENTREATH, V.W., TURNER, J.D. & COTTRELL, G.A. (1974). Effects of 6-hydroxydopamine on an identified dopamine-containing neuron in the central nervous system of *Planorbis corneus*. *Brain Res.*, **76**, 309–324.

CORRODI, H. & JONSSON, G. (1967). The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines. *J. Histochem. Cytochem.*, **15**, 65–78.

COTTRELL, G.A. (1970). Direct postsynaptic responses to stimulation of serotonin-containing neurons. *Nature, Lond.*, **225**, 1060–1062.

COTTRELL, G.A. (1971). Synaptic connections made by two serotonin-containing neurons in the snail (*Helix pomatia*) brain. *Experientia*, **27**, 813–815.

COTTRELL, G.A. & MACON, J.B. (1974). Synaptic connexions of two symmetrically placed giant serotonin-containing neurons. *J. Physiol.*, **236**, 435–464.

COTTRELL, G A & OSBORNE, N N (1970). Serotonin: Subcellular localisation in an identified serotonin neuron. *Nature, Lond.*, **225**, 470–472.

COTTRELL, G.A. & POWELL, B. (1971). Formation of serotonin by isolated serotonin-containing neurons and by isolated non-amine-containing neurons. *J. Neurochem.*, **18**, 1695–1697.

FALCK, B. & OWMAN, Ch (1965). A detailed methological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund Sec. II* No. 7, 1–23.

HAMBERGER, B., MAIMFORS, G. & SACHS, G. (1965). Standardisation of paraformaldehyde and of certain procedures for the histochemical demonstration of catecholamines. *J. Histochem. Cytochem.*, **13**, 147.

HORN, A.S, BAUMGARTEN, H.C & SCHLOSSBERGER, H.G. (1973). Inhibition of the uptake of 5-hydroxytryptamine, noradrenaline and dopamine into rat brain homogenates by various hydroxylated tryptamines. *J. Neurochem.*, **21**, 233–236.

KANDEL, E R. & TAUC, L (1966). Anomalous rectification in metacerebral giant cells and its consequences for synaptic transmission. *J. Physiol.*, **183**, 287–304.

LOWRY, O.H., ROSEBROUGH, N J, FARR, A.L. & RANDALL, R.J. (1951). Protein measurements with the folin-phenol reagent. *J. biol. Chem.*, **193**, 265–275.

MENG, K (1960). Untersuchung zur Störung der Herzatigkeit bei *Helix pomatia. Zool. Jahr*, **68**, 539–366.

McCAMAN, M.W., WEINREICH, D. & McCAMAN, R.E. (1973). The determination of picomole levels of 5-hydroxytryptamine and dopamine in *Aplysia, Tritonia* and Leech nervous tissues. *Brain Res.*, **53**, 129–137.

NEUHOFF, V (1973). Micro-determination of amino acids and related compounds with dansyl-chloride. In *Micromethods in molecular biology*, ed. Neuhoff, V., Vol. **14**, pp. 85–145.

OSBORNE, N.N. (1971). A microchromatographic method for the detection of biologically active monoamines in isolated neurons. *Experientia*, **27**, 1502–1503.

OSBORNE, N.N. (1972). The *in vivo* synthesis of serotonin in an identified serotonin-containing neuron of *Helix pomatia. Intern. J. Neuroscience*, **3**, 215–228.

OSBORNE, N N. (1973a). The analysis of amines and amino acids in micro-quantities of tissue. In *Progress in neurobiology*, Vol. **1**, part 4. eds. Kerkut, G.A. & Phillis, J.W., pp. 301–332. Oxford: Pergamon Press.

OSBORNE, N.N. (1973b). Micro-biochemical and physiological studies in the snail *Helix pomatia. Malacologia*, **14**, 97–106.

OSBORNE, N.N (1973c). Tryptophan metabolism in characterised neurones of *Helix. Br. J. Pharmac.*, **48**, 546–549.

OSBORNE, N.N. (1974a). Effect of 5,7-dihydroxytryptamine on the metabolism and accumulation of 5-hydroxytryptamine in the snail CNS. *Br. J. Pharmac.*, **52**, 152–153P.

OSBORNE, N N. (1974b). *Microchemical analysis of nervous tissue*. Oxford: Pergamon Press.

OSBORNE, N.N. & COTTRELL, G.A (1972). Amine and amino acid microanalysis of two identified snail neurons with known characteristics. *Experientia*, **28**, 656–658.

OSBORNE, N.N. & COTTRELL, G.A. (1971). Distribution of biogenic amines in the slug *Linex maximus. Z. Zellforsch.*, **112**, 15–30.

OSBORNE, N.N, HIRIPI, L. & NEUHOFF, V. (1975). The *in vitro* uptake of serotonin and catecholamines by snail (*Helix pomatia*) nervous tissue. *Biochem. Pharmac.* (in press).

OSBORNE, N.N & NEUHOFF, V. (1974). *In vitro* experiments on the metabolism, accumulation and release of 5-HT in the nervous system of the snail *Helix pomatia. J. Neurochem.*, **22**, 363–371.

PENTREATH, V.W. & COTTRELL, G A. (1972). Selective uptake of 5-hydroxytryptamine by axonal processes in *Helix pomatia. Nature, NavBiol.*, **239**, 213–214.

PENTREATH, V.W & COTTRELL, G.A. (1974). Anatomy of an identified serotonin neurone studied by means of injection of tritiated 'transmitter'. *Nature, Lond.*, **250**, 655–658.

(Received April 16, 1975.
Revised July 8, 1975)

Br. J. Pharmac. (1976), **56**, 39–41

BIOASSAY OF HISTAMINE IN THE PRESENCE OF PROSTAGLANDINS

R.J. GRYGLEWSKI & R. KORBUT
Department of Pharmacology, Copernicus Academy of Medicine, 16 Grzegorzecka, 31–531 Cracow, Poland

1 An Amberlite XAD-2 column in a heated (37°C) jacket was incorporated in between two banks of bioassay organs superfused with Krebs bicarbonate solution in cascade. The column removed prostaglandins E_1, E_2 and $F_{2\alpha}$ and also rabbit aorta contracting substance (RCS) and possibly slow reacting substance of anaphylaxis (SRS-A).

2 The column gave free passage to histamine in concentrations up to 3000 ng/ml. The method described improves the accuracy of histamine bioassay in the presence of prostaglandins.

Introduction

The technique of Vane (1964) has been used for a simultaneous bioassay of histamine, slow reacting substance of anaphylaxis (SRS-A), prostaglandins and rabbit aorta contracting substance (RCS) in the effluent from the shocked perfused lungs of sensitized guinea-pigs (Piper & Vane, 1969, 1971; Palmer, Piper & Vane, 1973; Piper, 1974). The cat terminal ileum (Ferreira, Ng & Vane, 1973) and the mepyramine-treated guinea-pig trachea were used to differentiate between histamine and SRS-A, and prostaglandins and RCS were bioassayed by standard procedures (Ferreira & Vane, 1967; Piper & Vane, 1969).

We have observed that the peak concentration of histamine released during anaphylactic shock from perfused guinea-pig lungs, as indicated by the difference between the responses of the antazoline-free and antazoline-treated guinea-pig ileum (1.08 μg/ml, s.e. ± 0.21, $n = 7$) is much higher than that determined spectrofluorimetrically by the method of Häkanson & Rönnberg (1974) (0.23 μg/ml, s.e. ± 0.04, $n = 7$). One of the reasons for this discrepancy may be the presence of prostaglandins and RCS in the effluent.

Here we describe a technique, which enables histamine to be bioassayed by the guinea-pig ileum in the presence of relatively high concentrations of prostaglandins.

Methods

Two banks of the assay organs, each consisting of a rabbit aortic strip, a rat stomach strip and a guinea-pig ileum, were superfused with Krebs bicarbonate solution of the following composition (mM): NaCl 118.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ $7H_2O$ 1.17, $CaCl_2$ $6H_2O$ 2.5, $NaHCO_3$ 25.0 and glucose 8.4, gassed with 95% O_2 and 5% CO_2. Krebs solution (37°C) was dripped over the organs at a rate of 2.5 ml/min, or perfused through the isolated lungs of sensitized guinea-pigs before reaching the organs (Piper & Vane, 1969). All substances were infused directly over the organs at a rate of 0.2 ml/min except for ovalbumin (10 mg), which was injected into the pulmonary artery. The combined antagonists (Gilmore, Vane & Wyllie, 1968), minus mepyramine, were infused directly over the tissues. The movements of the assay organs (initial load 2–3 g) were recorded with Paton's auxotonic levers connected to Harvard transducers (type 386) and a Watanabe multirecorder.

Amberlite XAD-2 (Keirse & Turnbull, 1973) or glass wool (in control experiments) were used to fill the column (2 × 5 cm). The column was maintained at 37°C by a water jacket and placed between the two banks of the assay tissues (Figure 1). Before being used the Amberlite column was washed twice with 100 ml portions of distilled water and then perfused with Krebs (2.5 ml/min) for 15 minutes.

The following substances were used: prostaglandins E_1, E_2 and $F_{2\alpha}$ (Upjohn Co.), histamine dihydrochloride (Polfa), antazoline methanesulphonate (Polfa), mepyramine maleate (May and Baker) and Amberlite XAD-2 (BDH Chemicals Ltd).

Results

In our experiments the threshold concentrations of prostaglandins E_1 and E_2 which contracted the rat stomach strip were 0.5–2 ng/ml, and those of prostaglandin $F_{2\alpha}$ were 2–10 ng/ml. The guinea-pig ileum was usually contracted by histamine in concentrations of 5–10 ng/ml, and also by prostaglandins E_1 and E_2

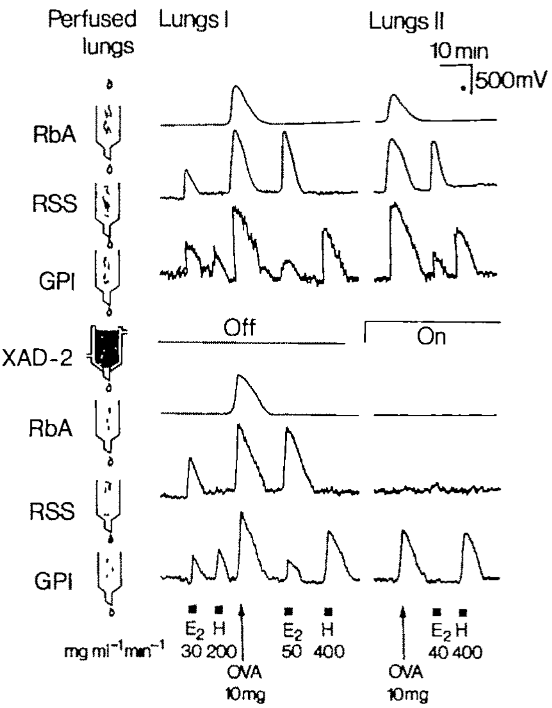


Figure 1 Isolated lungs of two sensitized guinea-pigs (lungs I and lungs II) were perfused with Krebs bicarbonate solution (2.5 ml/minute). The effluent was used to superfuse two banks of the assay tissues Each consisted of a rabbit aortic strip (RbA), a rat stomach strip (RSS), and a piece of guinea-pig ileum (GPI) All tissues were blocked with combined antagonists minus mepyramine (Gilmore *et al.*, 1968). Calibration amounts (■) of prostaglandin E_2 (E_2) and histamine (H) were infused directly into the effluent at the top of the cascade. Antigen (ovalbumin 10 mg) was injected into the pulmonary artery (OVA). During the perfusion of lungs II the XAD-2 column was placed between the two banks of tissues. The difference in the responses of the guinea-pig ilea above and below the column shows the influence of the interfering substances on histamine bioassay by the upper guinea-pig ileum.

in concentrations of 10–20 ng/ml. As partially exemplified in Figure 1, prostaglandins E_1, E_2 and $F_{2\alpha}$ at 10–150 ng/ml and also RCS from shocked guinea-pig lungs are reduced to undetectable levels during one passage through XAD-2. Amberlite absorbs neither histamine (10–3000 ng/ml) nor catecholamines (10–100 ng/ml). The guinea-pig ileum pretreated with antazoline (0.5 µg/ml) or with mepyramine (1.0 µg/ml) and situated beneath the XAD-2 column was contracted neither by histamine nor by the effluent from shocked lungs (5 experiments). It seems likely therefore that SRS-A is also absorbed by XAD-2.

Using the above method we have found that the effluent from shocked lungs of sensitized guinea-pigs contains histamine in the range 225–700 ng/ml (470 ± 54 ng/ml, mean ± s.e., $n = 10$) together with a prostaglandin-like substance (43.1 ± 3.7 ng/ml prostaglandin E_2 equivalents).

Discussion

An Amberlite XAD-2 column removes prostaglandins, RCS and possibly SRS-A, but not histamine from the effluent from the shocked lungs of sensitized guinea-pigs, and therefore the guinea-pig ileum situated distal to the column registers the actual amount of histamine in the effluent. A similar absorbent column, but filled with aluminium oxide, has been proposed by us for the bioassay of prostaglandins in the presence of high concentrations of catecholamines (Korbut, 1975; Grodzińska, Panczenko & Gryglewski, 1975).

Differential assay of biologically active substances in a mixture is possible with organ cascades because of the selective sensitivity of the assay organs towards individual hormones (Vane, 1964). This may be further improved by administration of combined antagonists (Gilmore *et al.*, 1968). The procedure described here provides another way of facilitating the specific bioassay of hormones and autacoids in mixtures.

We thank the Trustees of The Wellcome Trust (London) for the generous grant of equipment, and Dr J. Pike, Upjohn Co., Kalamazoo, U.S.A. for kindly supplying us with prostaglandins.

References

FERREIRA, S.H., NG, K.K. & VANE, J.R. (1973). The continuous bioassay of the release and disappearance of histamine in the circulation. *Br. J. Pharmac.*, **49**, 543–553.

FERREIRA, S.H. & VANE, J.R. (1967). Prostaglandins: their disappearance from and release into the circulation. *Nature, Lond.*, **216**, 868–873.

GILMORE, N, VANE, J.R. & WYLLIE, J.H. (1968). Prostaglandins released by the spleen. *Nature, Lond.*, **218**, 1135–1137.

GRODZIŃSKA, L., PANCZENKO, B. & GRYGLEWSKI, R.J. (1975). Generation of prostaglandin E-like material by the guinea pig trachea contracted by histamine. *J. Pharm. Pharmac.*, **27**, 88–91.

HÄKANSON, R. & RÖNNBERG, A.L. (1974). Improved fluorimetric assay of histamine: condensation with *o*-phthalaldehyde at −20°C. *Anal. Biochem.*, **60**, 560–567.

KEIRSE, M.J.N.C. & TURNBULL, A.C. (1973). Extraction of prostaglandins from human blood. *Prostaglandins*, **4**, 607–617.

KORBUT, R (1975). Bioassay of prostaglandins in the presence of high concentrations of catecholamines. *Pol. J. Pharmac. Pharm.* (in press).

PALMER, M.A., PIPER, P.J. & VANE, J.R. (1973). Release of rabbit aorta contracting substance (RCS) and prostaglandins induced by chemical or mechanical stimulation of guinea pig lungs. *Br. J. Pharmac.*, **49**, 226–242.

PIPER, P.J. & VANE, J.R. (1969). Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. *Nature, Lond.*, **223**, 29–35.

PIPER, PJ & VANE, J.R. (1971). The release of prostaglandins from lung and other tissues. *Ann. N.Y. Acad. Sci.*, **180**, 363–385.

PIPER, P.J. (1974). Release and metabolism of prostaglandins in lung tissue. *Pol. J. Pharmac. Pharm.*, **26**, 61–72.

VANE, J.R. (1964). The use of isolated organs detecting active substances in the circulating blood. *Br. J. Pharmac.*, **23**, 360–373.

(Received April 25, 1975.
Revised July 1, 1975)

Br. J. Pharmac. (1976), **56**, 43–48

TOLERANCE TO THE CARDIOVASCULAR EFFECTS OF Δ^9-TETRAHYDROCANNABINOL IN THE RAT

M.D. ADAMS, L.D. CHAIT & J.T. EARNHARDT
Department of Pharmacology, Health Sciences Division, Medical College of Virginia,
Virginia Commonwealth University, Richmond, Virginia, U.S.A. 23298

1 Daily intraperitoneal injections of Δ^9-tetrahydrocannabinol (Δ^9-THC, 10 mg/kg) resulted in tolerance to the effects of the cannabinoid on body weight and body temperature within 1–2 weeks of treatment.

2 Tolerance failed to develop to the suppression of spontaneous motor activity produced by Δ^9-THC during 28 days of treatment with the cannabinoid (10 mg/kg, i.p. per day).

3 Following treatment with vehicle for 28 days, intravenous administration of Δ^9-THC in anaesthetized rats produced a transient pressor response followed by a sustained hypotension and bradycardia.

4 Tolerance to the hypotensive and negative chronotropic responses to intravenous Δ^9-THC was readily apparent in animals which had received daily intraperitoneal injections of Δ^9-THC (10 mg/kg) for 28 days.

5 Tolerance failed to develop to the pressor actions of intravenous Δ^9-THC after 28 days of pretreatment.

6 There was no difference in the pressor response to intravenous noradrenaline in vehicle-treated animals (1.0 ml/kg, i.p., per day for 28 days) and Δ^9-THC-treated animals (10 mg/kg, i.p., per day for 28 days).

Introduction

Tolerance to the effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) or extracts of marihuana has been demonstrated by several investigators in a variety of experimental situations in laboratory animals. McMillan, Harris, Frankenheim & Kennedy (1970) demonstrated a reduction in the inhibitory effect of Δ^9-THC on key pecking behaviour in the pigeon. Others have shown tolerance to the effects of Δ^9-THC on bar pressing behaviour in rats and pigeons (Ford & McMillan, 1971), on overt behaviour (static ataxia) in the dog (Dewey, Jenkins, O'Rourke & Harris, 1972), on the conditioned avoidance response (Bailey, Pradhan & Ghosh, 1971), on body weight (Graham & Li, 1973), on locomotor activity (Davis, Moreton, King & Pace, 1972), on body temperature in the rat (Sofia, 1972), and on locomotor activity and body temperature in the neonatal chick (Abel, 1972).

Studies designed to determine whether tolerance develops to the cardiovascular actions of Δ^9-THC have been contradictory and inconclusive. Graham & Li (1973) demonstrated a reduction in the hypotensive and negative chronotropic effects of an intravenously administered extract of cannabis in rats following 14 days of pretreatment with the extract. Also, Nahas, Schwartz, Adamec & Manger (1973) presented evidence for the rapid development of tolerance to the hypotensive effects of Δ^9-THC in spontaneously hypertensive rats. However, studies by Birmingham (1973), Williams, Ng, Lamprecht, Roth & Kopin (1973) and Lewis, Brown & Forney (1974), failed to demonstrate tolerance to the hypotensive actions of Δ^9-THC in either hypertensive (spontaneous or adrenal-regeneration) or normotensive animals following pretreatment periods ranging from 7–14 days.

Recent studies in this laboratory indicate that intraarterially administered Δ^9-THC produces vasoconstriction in the perfused hind-quarters of the rat (Adams, Dewey & Harris, 1974). Furthermore, it was observed that intravenously administered Δ^9-THC produced transient, dose-related pressor responses that were followed by the characteristic hypotensive and negative chronotropic effects. Therefore, this study was undertaken to determine if pretreatment with Δ^9-THC produces tolerance to the pressor, depressor, and negative chronotropic effects of intravenously administered Δ^9-THC in normotensive rats. In addition, pressor responses to noradrenaline were evaluated in the light of recent data suggesting that Δ^9-THC might act as an adrenergic neurone blocking agent (Graham, Lewis & Li, 1974). Effects of Δ^9-THC treatment on body weight, spontaneous motor activity, and body temperature, were examined during the course of the treatment schedule to assess tolerance development.

Methods

Male, Sprague-Dawley rats (Flow Laboratories, Dublin, Virginia, U.S.A.) with initial body weights ranging from 245–270 g were used for this study. Animals were housed in individual cages in temperature-controlled facilities (21–22°C) with an alternating 12 h dark-light cycle. Animals had access to standard laboratory chow and drinking water *ad libitum*.

Treatment schedules

Sixteen rats were randomly assigned to two groups designated as vehicle-treated and Δ^9-THC-treated, respectively. Δ^9-THC-treated animals received daily, intraperitoneal injections of 10 mg/kg Δ^9-THC (solubilized in an ethanol: Emulphor EL-620: saline vehicle) for 28 days. Vehicle-treated animals received daily injections of equivalent volumes, on a weight basis, of vehicle alone for 28 days. Body weight was recorded daily at the time of injection with all injections made between 11 h 00 min and 13 h 15 min. During the course of the treatment schedule, two animals from the Δ^9-THC group and one animal from the vehicle-control group were killed because of the appearance of symptoms indicative of a respiratory tract infection.

Measurement of body temperature

Body temperature was measured with a rectal probe and a telethermometer (Model 43-T3, Yellow Springs Instrument Company, Yellow Springs, Ohio, U.S.A.). The hypothermic response to Δ^9-THC was evaluated on days 1, 5, 12, 20 and 28 of the treatment schedule by recording the temperature of all animals, before and 70 min after the administration of either Δ^9-THC or the vehicle. Data were expressed as the change (°C) from the pre-injection value.

Measurement of spontaneous motor activity

Spontaneous motor activity was measured on a Lafayette Activity Platform (Lafayette Instrument Company, Lafayette, Indiana, U.S.A.) which records all types of movement. Total activity (counts) was recorded for 10 min periods during each testing session. Spontaneous motor activity was evaluated 60 min after injection of either Δ^9-THC or vehicle on days 1, 8, 15, 20 and 28 of the treatment schedule.

Measurement of blood pressure and heart rate

On the day following the final injection of either Δ^9-THC or vehicle, all animals were anaesthetized with urethane (1.2 g/kg, i.p.) and prepared for intravenous drug administration and measurement of blood pressure and heart rate. After insertion of a tracheal cannula, a polyethylene cannula (PE-20) was placed in the right external jugular vein for drug administration. The left carotid artery was cannulated (PE-90) and blood pressure monitored by a Statham p-23A transducer and recorded on a Grass Model 7B polygraph. The mean arterial pressure was calculated as follows: mean arterial pressure (mmHg) = diastolic pressure + 1/3 (systolic pressure − diastolic pressure). Heart rate was determined from the blood pressure tracing. Drugs were administered in the jugular cannula in 0.1 ml volumes and flushed with 0.2 ml of saline (0.9% w/v NaCl solution).

After a 5 min equilibration period, each animal received noradrenaline 0.3 μg/kg and the pressor response was determined. After blood pressure returned to the control value, each animal received an injection of the vehicle used for Δ^9-THC and blood pressure was recorded during the first minute and 3, 5 and 10 min after vehicle administration. Heart rate was recorded 3, 5 and 10 min after vehicle administration. Finally each animal received Δ^9-THC 1.0 mg/kg, blood pressure was recorded during the first minute after injection and both blood pressure and heart rate were recorded at 3, 5, 10, 20, 30 and 40 min after administration of Δ^9-THC.

Drugs

Δ^9-THC was prepared as a solution (100 mg/ml) in a 1 : 1 mixture of absolute ethanol and a polyoxyethylated vegetable oil (Emulphor EL-620; GAF Corporation, New York, New York, U.S.A.). This stock solution was diluted with saline to yield appropriate concentrations such that 0.1 ml would deliver a dose of 1.0 mg/kg. Similar concentrations of vehicle were employed as control injections. Noradrenaline bitartrate (Levophed; Winthrop Laboratories, New York, New York, U.S.A.) was diluted with saline such that 0.1 ml would provide a dose of 0.3 μg/kg of the free base.

Statistics

Statistical tests employed in the analysis of data include Student's *t* test, paired *t* test, and a multivariate profile analysis described by Morrison (1967). Values of $P < 0.05$ were considered significant.

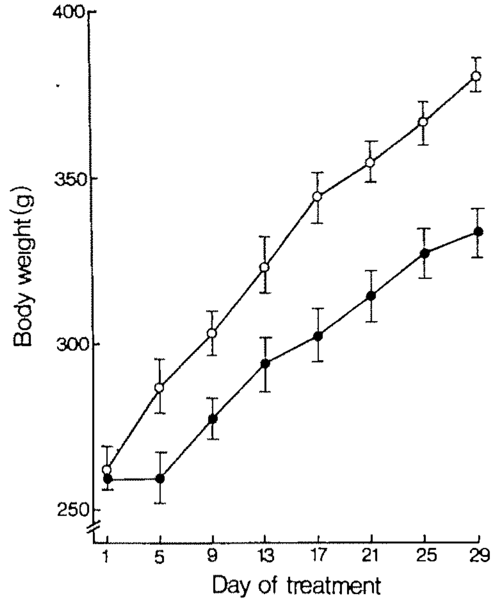


Figure 1 Effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) on body weight. Rats treated daily with vehicle 1 mg/kg, i.p. (○); rats treated daily with Δ^9-THC, 10 mg/kg, i.p. (●). Each point represents the mean of results from 6–8 animals. Vertical lines represent s.e. mean. Lines between days 1 and 5 are not parallel ($P<0.002$); while segments between days 5 and 29 are parallel ($P>0.14$)

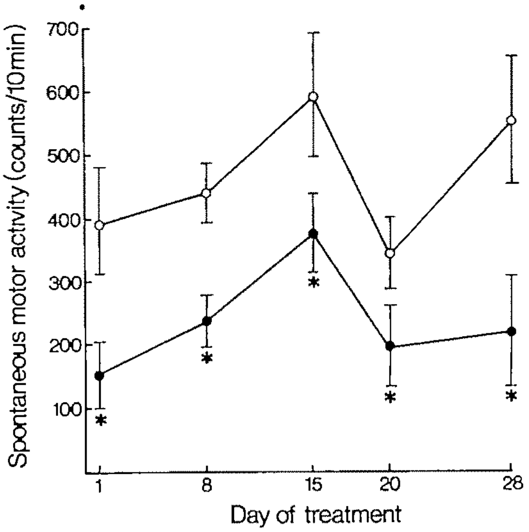


Figure 2 Effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) on spontaneous motor activity Rats treated daily with vehicle, 1 ml/kg, i.p. (○); rats treated daily with Δ^9-THC, 10 mg/kg, i.p. (●). Each point represents the mean of results from 6–8 animals Vertical lines represent s.e. mean.

* Significant difference between groups (minimum $P<0.05$).

Results

Effects of Δ^9-THC on body weight

The effects of daily injections of Δ^9-THC on body weight are illustrated in Figure 1. Body weights were recorded daily but for clarity and statistical evaluation only the data shown were employed. Vehicle-treated animals exhibited a normal growth curve and gained weight throughout the course of the experiment. Δ^9-THC-treated animals failed to gain weight during the first five days of treatment and weighed significantly less than vehicle-treated animals on day 5. However, from day 5 to the end of the treatment period, Δ^9-THC-treated animals gained weight at the same rate as vehicle-treated animals, indicating that tolerance to the depressant effects of Δ^9-THC on body weight had developed.

Effects of Δ^9-THC on spontaneous motor activity

Figure 2 clearly illustrates that under the conditions of this experiment, tolerance did not develop to the effects of Δ^9-THC on spontaneous motor activity. On all days in which effects of Δ^9-THC on spontaneous motor activity were examined, there were significant differences in activity between control and Δ^9-THC-treated rats.

Effects of Δ^9-THC on body temperature

Initial treatment (day 1) with Δ^9-THC (10 mg/kg, i.p.) produced a marked decrease in body temperature as indicated in Figure 3. Significant differences in the body temperatures of vehicle- and Δ^9-treated animals were still apparent on days 5 and 8 of treatment, although the magnitude of the hypothermic response was significantly reduced when compared to day 1. On day 12, there was no significant hypothermic response in the Δ^9-THC-treated rats but a slight hypothermia was noted on day 15. However, on days 20 and 28, there were no significant differences in body temperature responses to vehicle or Δ^9-THC, indicating tolerance to the hypothermic actions of Δ^9-THC.

Effects of Δ^9-THC-pretreatment on the blood pressure responses to intravenously administered noradrenaline and Δ^9-THC

Initial mean arterial pressures in the anaesthetized animals were 79.0 and 78.5 mmHg, respectively, for

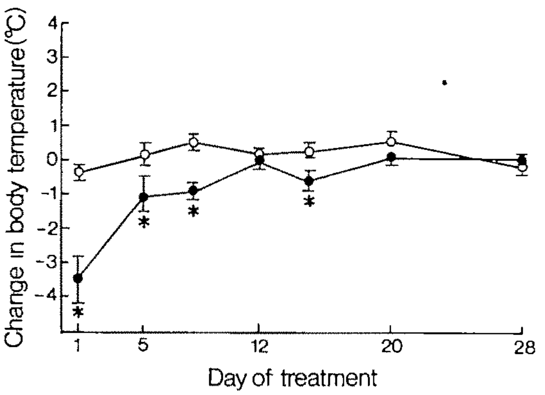


Figure 3 Effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) on body temperature. Rats treated daily with vehicle, 1 ml/kg, i.p. (○); rats treated daily with Δ^9-THC, 10 mg/kg, i.p (●). Each point represents the mean of results from 6–8 animals. Vertical lines represent s.e. mean.
* Significant difference between groups (minimum $P<0.05$).

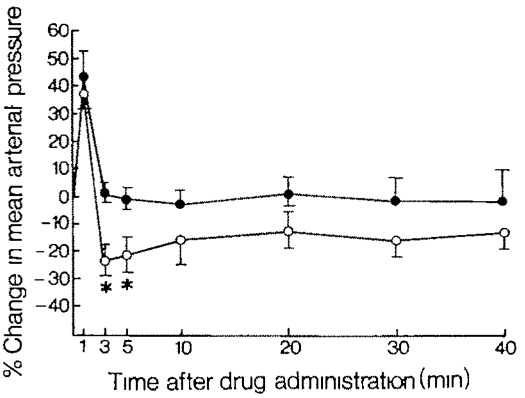


Figure 4 Effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) pretreatment on the blood pressure response to intravenously administered Δ^9-THC (1.0 mg/kg) Rats receiving 28 daily injections of vehicle, 1 ml/kg, i.p (○), rats treated daily with Δ^9-THC, 10 mg/kg, i.p. (●). Each point represents the mean of results from 6 animals. Vertical lines represent s.e. mean
* Significant difference between groups (minimum $P<0.05$).

vehicle-pretreated and Δ^9-THC-pretreated animals. There was no significant difference in the maximum pressor response to noradrenaline (0.3 µg/kg, i.v.) in vehicle-pretreated and Δ^9-THC pretreated rats (49.2 as compared with 40.7 mmHg).

In animals pretreated for 28 days with vehicle, the intravenous administration of Δ^9-THC produced the biphasic effect on blood pressure (Figure 4) previously observed in anaesthetized rats (Adams *et al.*, 1974). Following a transient increase in pressure, there was a more sustained hypotensive effect. In the Δ^9-THC-pretreated animals, intravenous Δ^9-THC produced a pressor response equivalent to that seen in the vehicle-pretreated group. However, the hypotensive phase of the blood pressure response was absent in the Δ^9-THC pretreated animals indicating development of tolerance to this action of Δ^9-THC. Intravenous injections of vehicle produced small, transient increases in pressure which were not dose-related and were possibly due to the volume of injection. This vehicle pressor response was subtracted from the drug-induced pressor response in each animal. The vehicle did not have hypotensive activity.

Effects of Δ^9-THC pretreatment on the heart rate response to intravenously administered Δ^9-THC

Initial heart rates did not differ significantly in vehicle-pretreated and Δ^9-THC-pretreated animals (268.3 as compared with 304.0). The negative chronotropic effect of acutely administered Δ^9-THC was reduced significantly by the 28 days pretreatment with Δ^9-THC as shown in Figure 5. However, in contrast to the complete absence of the hypotensive response, Δ^9-THC did produce bradycardia (10–15%) in these animals. Intravenous administration of the vehicle failed to alter heart rate in either group of animals.

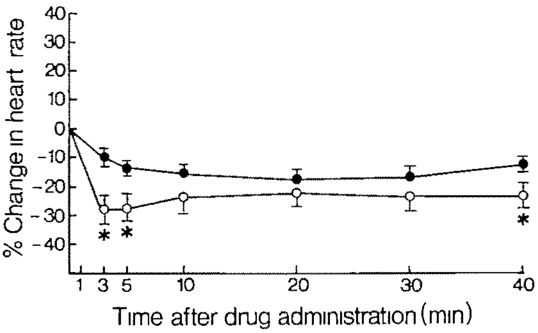


Figure 5 Effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) pretreatment on the heart rate response to intravenously administered Δ^9-THC (1.0 mg/kg). Rats receiving 28 daily injections of vehicle, 1 ml/kg, i.p (○); rats treated daily with Δ^9-THC, 10 mg/kg, i.p. (●). Each point represents the mean of results from 6 animals. Vertical lines represent s.e. mean
* Significant difference between groups (minimum $P<0.05$)

Discussion

These studies confirm that tolerance develops to the effects of Δ^9-THC on body weight and body temperature in the rat. Food consumption was not measured in this study; however, it was assumed that the failure of Δ^9-THC-treated animals (10 mg/kg per day, i.p.) to gain weight during the first 4 days of treatment could be attributed to the anorexic effect reported by a number of investigators (Manning, McDonough, Jr, Elsmore, Saller & Sodetz, 1971; Jarbe & Henriksson, 1973; Sofia & Barry, 1974). Tolerance to the effects of Δ^9-THC on body weight and body temperature developed within 5 days during the daily treatment regimen.

Rats failed to exhibit tolerance to the depressant effects of Δ^9-THC 10 mg/kg on spontaneous motor activity during 28 days of treatment. This is in contrast to the report by Davis *et al.* (1972) who demonstrated tolerance to the suppression of spontaneous motor activity after 11 days of daily intraperitoneal injections of 25 mg/kg of Δ^9-THC. In addition to the dosage differences, there are other variations in the experimental procedures (drug vehicles, recording devices, duration of recording sessions, etc.) which rule out direct comparisons between this study and that of Davis and co-workers.

Following 28 days of pretreatment with Δ^9-THC, there was clearly tolerance to the hypotensive and negative chronotropic effects of intravenously administered Δ^9-THC in anaesthetized rats. The characteristic hypotensive response to Δ^9-THC was abolished by the Δ^9-THC pretreatment regimen and the reduction in heart rate was significantly less (40–50%) than in vehicle-pretreated animals. These data are consistent with the previous report by Graham & Li (1973) who demonstrated tolerance in rats, to the hypotensive and negative chronotropic effects of an intravenously administered extract of cannabis following 14 days of treatment with that extract. However, Lewis *et al.* (1974) found no evidence of tolerance to the hypotensive action of Δ^9-THC in spontaneously hypertensive rats after 9 days of treatment with up to 10 mg/kg per day of Δ^9-THC.

Recently, Graham *et al.* (1974) presented evidence that Δ^9-THC could interfere with the release of noradrenaline during transmural stimulation of the rat isolated vas deferens. These investigators concluded that Δ^9-THC may have adrenergic neurone blocking properties. Because of the well-documented ability of pretreatment with adrenergic neurone blocking agents to elicit supersensitivity to exogenous noradrenaline (Burn & Rand, 1958), the pressor actions of intravenous noradrenaline were examined in rats pretreated with Δ^9-THC or vehicle for 28 days. There was, however, no indication of supersensitivity to noradrenaline in Δ^9-THC pretreated animals as compared to vehicle pretreated controls.

As previously reported (Adams *et al.*, 1974), intravenously administered Δ^9-THC elicits a biphasic blood pressure response in the anaesthetized rat. This response consists of a transient pressor phase followed by a more sustained reduction in blood pressure which is accompanied by bradycardia. Furthermore, the intraarterial injection of Δ^9-THC into the perfused hindquarters of the rat produces dose-related constrictor responses. This suggests that the initial pressor phase following intravenous administration may be the result of an increase in peripheral resistance. The current study clearly shows that tolerance to the pressor effect of Δ^9-THC did not develop during this treatment regimen. As previously stated, Δ^9-THC pretreated animals did exhibit a reduction in heart rate (10–15%) following acute administration of Δ^9-THC in the absence of any hypotensive response. This would indicate that an increase in stroke volume and/or an increase in peripheral resistance is acting to offset the reduction in blood pressure that might result from the bradycardia.

In conclusion, these studies clearly demonstrate that tolerance develops to the hypotensive and negative chronotropic effects of Δ^9-THC in rats previously shown to be tolerant to the body weight and hypothermic effects of the compound. However, there was no tolerance to the transient pressor activity of Δ^9-THC. The hypothermia, reduced growth rate, hypotension, and bradycardia produced by Δ^9-THC are probably manifestations of central nervous system actions (Hardman, Domino & Seevers, 1957; Manning *et al.*, 1971; Lomax, 1971; Hardman, Domino & Seevers, 1971; Cavero, Buckley & Jandhyala, 1973a; Cavero, Solomon, Buckley & Jandhyala, 1973b) while previous studies in this laboratory suggest that the pressor response is a peripheral vascular effect. Thus, it would appear that while tolerance to actions of Δ^9-THC in the central nervous system can readily be demonstrated, there is no tolerance to what appears to be a peripheral effect of Δ^9-THC. This is analagous to the absence of tolerance to the effects of morphine on intestinal smooth muscle at a time when tolerance to the CNS effects of the narcotic can easily be demonstrated.

This study was supported in part by Grants DA-00490 and T32-GM-07111 from the United States Public Health Service.

References

ABEL, E.L. (1972). Studies of tolerance to 1-Δ^9-tetrahydrocannabinol (Δ^9-THC) in neonatal chicks. *Fedn Proc.*, **31**, 505 Abstract.

ADAMS, M.D., DEWEY, W.L. & HARRIS, L.S. (1974). Cardiovascular effects of Δ^8- and Δ^9-tetrahydrocannabinol in rats. *Pharmacologist*, **16**, 260 Abstract.

BAILEY, P.T., PRADHAN, S.N. & GHOSH, B. (1971). Some behavioural effects of Δ^9-tetrahydrocannabinol (Δ^9-THC). *Fedn Proc.*, **30**, 279 Abstract.

BIRMINGHAM, M.K. (1973). Reduction by Δ^9-tetrahydrocannabinol in the blood pressure of hypertensive rats bearing regenerated adrenal glands. *Br. J. Pharmac.*, **48**, 169–171.

BURN, J.H. & RAND, M.J. (1958). The action of sympathomimetic amines in animals treated with reserpine. *J. Physiol.*, **144**, 314–336.

CAVERO, I., BUCKLEY, J.P. & JANDHYALA, B.S. (1973a). Hemodynamic and myocardial effects of (–)-Δ^9-trans-tetrahydrocannabinol in anesthetized dogs. *Eur. J. Pharmac.*, **24**, 243–251.

CAVERO, I., SOLOMON, T., BUCKLEY, J.P. & JANDHYALA, B.S. (1973b). Studies on the bradycardia induced by (–)-Δ^9-trans-tetrahydrocannabinol in anesthetized dogs. *Eur. J. Pharmac.*, **22**, 263–269.

DAVIS, W.M., MORETON, J.E., KING, W.T. & PACE, H.B. (1972). Marihuana and locomotor activity: biphasic effect and tolerance development. *Res. Commun. Chem. Path. Pharmac.*, **3**, 29–35.

DEWEY, W.L., JENKINS, J., O'ROURKE, T. & HARRIS, L.S. (1972). The effects of chronic administration of trans-Δ^9-tetrahydrocannabinol on behavior and the cardiovascular system of dogs. *Arch. int. Pharmacodyn. Ther.*, **198**, 118–131.

FORD, R.D. & McMILLAN, D.E. (1971). Behavioural tolerance and cross tolerance to 1-Δ^8-tetrahydrocannabinol (Δ^8-THC) and 1-Δ^9-tetrahydrocannabinol (Δ^9-THC) in pigeons and rats. *Fedn. Proc.*, **30**, 279 Abstract.

GRAHAM, J.D.P. & LI, D.M.F. (1973). Cardiovascular and respiratory effects of cannabis in cat and rat. *Br. J. Pharmac.*, **49**, 1–10.

GRAHAM, J.D.P., LEWIS, M.J. & LI, D.M.F. (1974). The effect of Δ^1-tetrahydrocannabinol on the release of [^{3}H]-(1)-noradrenaline from the isolated vas deferens of the rat. *Br. J. Pharmac.*, **52**, 233–236.

HARDMAN, H.F., DOMINO, E.F. & SEEVERS, M.H. (1957). The chemistry and pharmacology of certain compounds affecting the central nervous system of animals and man. *Clearinghouse for Federal Scientific and Technical Information*, AD 707–699, pp. 1–53.

HARDMAN, H.F., DOMINO, E.F. & SEEVERS, M.H. (1971). General pharmacological actions of some synthetic tetrahydrocannabinol derivatives. *Pharmacol. Rev.*, **23**, 295–315.

JARBE, T.U.C. & HENRIKSSON, B.G. (1973). Acute effects of two tetrahydrocannabinols (Δ^9-THC and Δ^8-THC) on water intake in water deprived rats: implications for behavioral studies on marijuana compounds. *Psychopharmacologia*, **30**, 315–322.

LEWIS, S.C., BROWN, D.J. & FORNEY, R.B. (1974). Absence of tolerance to the hypotensive effects of delta-9-tetrahydrocannabinol in hypertensive rats. *Toxicol. Appl. Pharmac.*, **28**, 7 Abstract.

LOMAX, P. (1971). Acute tolerance to the hypothermic effect of marihuana in the rat. *Res. Commun. Chem. Path. Pharmac.*, **2**, 159–167.

McMILLAN, D.E., HARRIS, L.S., FRANKENHEIM, J.M & KENNEDY, J.S (1970). I-Δ^9-trans-tetrahydrocannabinol in pigeons: tolerance to the behavioral effects. *Science*, **169**, 501–503.

MANNING, F.J., McDONOUGH, J.H., Jr., ELSMORE, T.F., SALLER, C. & SODETZ, F.J. (1971). Inhibition of normal growth by chronic administration of Δ^9-tetrahydrocannabinol. *Science*, **174**, 424–426.

MORRISON, D.F. (1967). *Multivariate statistical methods*. New York, N.Y.: McGraw-Hill Publishing Co.

NAHAS, G.G., SCHWARTZ, I.W., ADAMEC, J. & MANGER, W.M. (1973). Tolerance to delta-9-tetrahydrocannabinol in the spontaneously hypertensive rat. *Proc. Soc. exp. Biol. Med.*, **142**, 58–60.

SOFIA, R.D. (1972). A paradoxical effect for Δ^1-tetrahydrocannabinol on rectal temperature in rats. *Res. Commun.Chem. Path. Pharmac.*, **4**, 281–288.

SOFIA, R.D. & BARRY, H. (1974). Acute and chronic effects of Δ^9-tetrahydrocannabinol on food intake by rats. *Psychopharmacologia*, **39**, 213–222.

WILLIAMS, R.B., NG, L.K.Y., LAMPRECHT, F., ROTH, K. & KOPIN, I.J. (1973). Δ^9-tetrahydrocannabinol: a hypotensive effect in rats. *Psychopharmacologia*, **28**, 269–274.

(Received May 3, 1975)

Br. J. Pharmac. (1976), **56**, 49–57

EFFECTS OF ANTICONVULSANT AND CONVULSANT DRUGS ON THE ATPASE ACTIVITIES OF SYNAPTOSOMES AND THEIR COMPONENTS

J.C. GILBERT & M.G. WYLLIE

Department of Pharmacology, University of Aberdeen, Foresterhill, Aberdeen, AB9 2ZD

1 The effects of anticonvulsants, and other drugs on the Na^+,K^+-adenosine triphosphatase (ATPase) (ouabain-sensitive) and Mg^{++}-ATPase activities of synaptosomes and their components have been determined.

2 The Mg^{++}-ATPase activity of synaptosomes was not affected by the drugs but the Na^+,K^+-ATPase activity was inhibited by phenytoin (diphenylhydantoin), ethosuximide and diazepam.

3 Fractions containing mainly membranes, mitochondria or synaptic vesicles, were prepared from synaptosomes by osmotic shock and subsequent density gradient centrifugation. Inhibition of Na^+,K^+-ATPase activity by phenytoin, ethosuximide and diazepam was apparent only in the membrane fraction.

4 The fraction containing synaptic vesicles exhibited pronounced Mg^{++}-ATPase but no Na^+,K^+-ATPase activity. In contrast to the enzymes of the membranes and mitochondria, the Mg^{++}-ATPase of the vesicles was inhibited by diazepam and all of the anticonvulsants tested.

Introduction

Mechanisms of action of anticonvulsant drugs are still a matter for speculation and no one effect of the drugs stands out sufficiently to support the contention that one mechanism of anticonvulsant action is common to all members of the group. The theory that the increase in the glucose concentration of the brain caused by all the anticonvulsants tested is involved in their action through membrane stabilization (Gilbert, Gray & Heaton, 1971) has received support (Nahorski, 1972) but it is a difficult one to test. Of the other effects of the drugs the changes which some induce in brain sodium, potassium-activated, magnesium-dependent adenosine triphosphatase (Na^+,K^+-ATPase, EC.3.6.1.3) activity, thereby influencing ion gradients, are of particular interest. However, whilst there is little doubt that phenytoin (diphenylhydantoin) can influence brain Na^+,K^+-ATPase activity, whether inhibition or stimulation results appears to depend upon the concentrations of sodium and potassium ions in the assay medium (Festoff & Appel, 1968; Rawson & Pincus, 1968; Formby, 1970; Woodbury & Kemp, 1970; Gilbert, Buchan & Scott, 1974a; Gilbert & Wyllie, 1974b; Koostra & Woodhouse, 1974). The anticonvulsant ethosuximide also inhibits Na^+,K^+-ATPase but previous experiments in this laboratory have failed to detect effects of phenobarbitone or acetazolamide on the activity of the enzyme (Gilbert *et al.*, 1974a).

The suggestion of Paton, Vizi & Zar (1971) that acetylcholine release in the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum is regulated through inhibition of Na^+,K^+-ATPase has added new significance to the observations concerning phenytoin action. Recent experiments (Gilbert, Wyllie & Davison, 1975) have provided data supporting this interesting hypothesis but the precise relationships between the activities of the several ATPases of the nerve terminal and transmitter release have yet to be established.

In this paper we describe experiments to determine effects of anticonvulsants, convulsants and other drugs on the magnesium-activated ATPase (Mg^{++}-ATPase) and Na^+,K^+-ATPase activities of synaptosomes prepared from rat cerebral cortex. An attempt has also been made to define the sites of the effects more precisely within the synaptosome by examining separately the mitochondria, membranes and synaptic vesicles prepared from synaptosomes by osmotic disruption and density-gradient centrifugation.

Methods

Male Sprague-Dawley rats weighing 250–450 g were used, the preparation of synaptosomes usually involving eight animals. They were stunned and

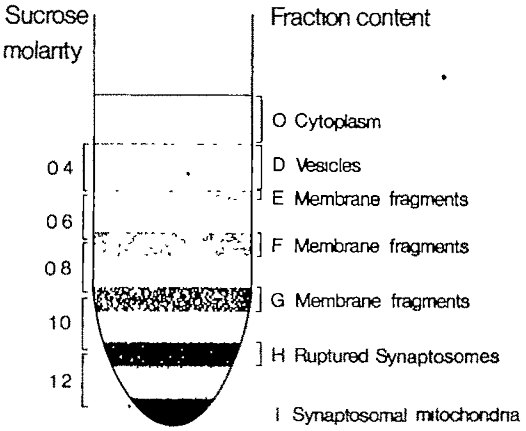


Figure 1 The synaptosome pellet was homogenized in distilled water (2 ml/g of original cortex) and separated on a sucrose gradient. The diagram represents the appearance of the centrifuge tube after centrifugation at 53,500 *g* for 2 hours.

decapitated and the cerebral cortices rapidly removed and cleared of white matter. All subsequent operations from the preparation of the cortex homogenate to the recovery of the synaptosomal and other fractions were performed at 0–4°C. The cortices were homogenized in 0.32 M sucrose containing 1 mM ethylenediamine tetraacetic acid (EDTA) using a Potter-Elvehjem homogenizer fitted with a teflon pestle; the clearance was 0.23 mm and the speed of rotation 840 rev/minute.

The homogenate (10%) was separated into the various primary fractions by a modification of the method of Gray & Whittaker (1962). The nuclear, pellet and the crude mitochondrial fraction were spun down at 1000 *g* (10 min) and 10,000 *g* (20 min) respectively, and the synaptosomes were recovered from the mitochondrial fraction by resuspending it in sucrose/EDTA solution (2–3 ml/g original cortex) and centrifuging it at 53,500 *g* for 2 h on a sucrose density gradient (5 ml 1.2 M and 7.5 ml 0.8 M sucrose solution). The synaptosome suspension was removed from between the 0.8 and 1.2 M sucrose solution, the molarity of the sucrose adjusted to 0.4 M and the suspension centrifuged at 100,000 *g* for 30 min to obtain the synaptosome pellet. For studies of synaptosome components the pellet was disrupted by homogenization in distilled water (2 ml/g original cortex) and standing at room temperature for 60 minutes. This suspension was placed on a discontinuous density gradient consisting of successive 3 ml layers of sucrose (Figure 1) as described by Whittaker, Michaelson & Kirkland (1964).

After centifuging at 53,500 *g* for 2 h the bands were removed separately with a needle attached to a 20 ml syringe. The seven fractions obtained were designated O, D, E, F, G, H and I in order of the increasing density of sucrose and these corresponded to the cytoplasm (O), vesicle (D), membrane (E, F, G), ruptured synaptosomes (H) and mitochondria (I) fractions from synaptosomes.

Samples of the fractions were stored at −23°C before determination of the protein contents or enzymatic activities. Results obtained with freshly prepared material were not altered significantly by storage at this temperature.

ATP (disodium salt; Sigma Chemical Company) was converted into the free acid by passing it through Dowex 50 resin (H$^+$ form, 200–400 mesh) in a cold room and adjusting it to pH 7.40 with 1 M tris solution. Deionized water was added to make the solution 40 mM with respect to ATP, the final concentration being calculated from the extinction of the solution at 260 nm. The concentration of sodium ions in this solution was less than 100 µM and was less than 10 µM in the final ATPase assay medium.

The concentrations of endogenous sodium and potassium ions were determined in some preparations by flame photometry and by atomic absorption spectrophotometry after digestion of samples overnight in 15.8 N nitric acid and subsequent dilution.

For ATPase assays the tissue fractions were resuspended in 50 mM imidazole/HCl buffer pH 7.40 and 0.2 ml samples, each containing 0.1–0.2 mg protein, were added to buffered media. The media contained NaCl (150 mM), KCl (10 mM) and MgCl$_2$ (5 mM) for total ATPase, MgCl$_2$ (5 mM) for Mg^{++}-ATPase and MgCl$_2$ (5 mM) and NaCl (150 mM) for Na$^+$-ATPase (see below) assays. The mixtures (final volume 0.9 ml) were pre-incubated for 15 min at 37°C before starting the reactions by addition of 0.1 ml Tris ATP solution (4 mM final concentration). Sodium dodecyl sulphate (1 ml, 0.8%) was used to stop the reactions after 10 minutes. The phosphate contents of the clear solutions were determined by the method of Bonting, Simon & Hawkins (1961). When used, drugs were added to the pre-incubation media. Na$^+$,K$^+$-ATPase activity was calculated by subtracting the Mg^{++}-ATPase activity from the total ATPase activity.

We have recently shown (Gilbert & Wyllie, 1974a; 1975) that the Na$^+$,K$^+$-ATPase activity so calculated (that is, relative to the total activity determined in the presence of sodium, potassium and magnesium ions) consists of at least two components. One of these is not inhibited by ouabain and appears to be due to a sodium-activated, magnesium-dependent ATPase (Na$^+$-ATPase). This component contributes some 25–30% to the activity of the apparent Na$^+$,K$^+$-ATPase in synaptosomes from rat cerebral cortex and in the present study none of the drugs tested influenced its activity. For simplicity, therefore, we have reported here the activity pertaining to the Na$^+$,K$^+$-ATPase

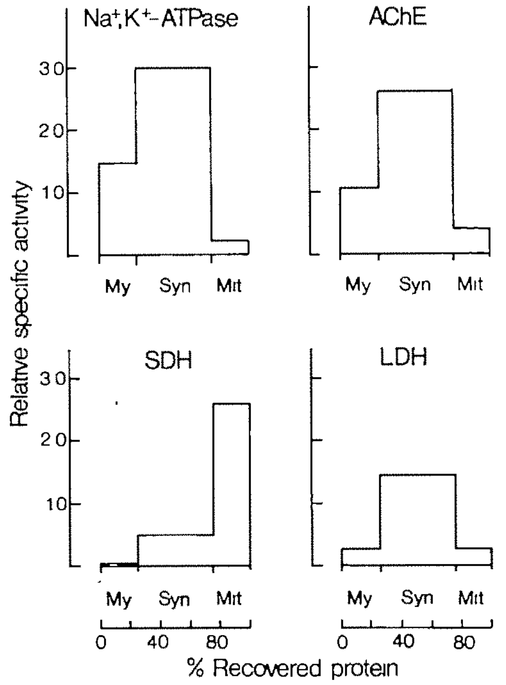


Figure 2 Distribution of the cytoplasmic marker lactate dehydrogenase (LDH), the mitochondrial marker succinate dehydrogenase (SDH), and two membrane fragment markers Na$^+$,K$^+$-ATPase and acetylcholinesterase (AChE). The crude mitochondrial fraction was separated into myelin (My), synaptosomes (Syn) and mitochondria (Mit) by density gradient centrifugation. The ordinates (relative specific activity) are the percentages of the enzymes recovered in each fraction divided by the percentages of protein recovered in the same fraction.

(EC.3.6.1.3), the enzyme which requires sodium and potassium ions together for activation in the presence of magnesium ions. The activity was calculated by subtraction of the Mg^{++}-ATPase activity from the total ATPase activity.

The protein contents of solutions were determined by the method of Lowry, Rosenbrough, Farr & Randall (1951). Acetylcholinesterase was assayed manometrically by the method of Aldridge & Johnson (1959). Succinate dehydrogenase was assayed by the manometric technique of Quaestel & Wheatley (1938) as described and modified by Aldridge & Johnson (1959). Lactate dehydrogenase was assayed spectrophotometrically (Johnson, 1960).

The following drugs were used: acetazolamide sodium (Lederle); barbitone sodium (BDH); diazepam (Roche Products); dimethadione (Abbot Laboratories); ethosuximide (Parke Davis); Leptazol (Sigma); ouabain (BDH); phenobarbitone sodium (BP); phenytoin sodium (Sigma); picrotoxin (BDH); SC-13504 (Searle); strychnine sulphate (Sigma); trimethadione (Abbot Laboratories).

Results

The distribution of the enzyme markers Na$^+$,K$^+$-ATPase, acetylcholinesterase, succinate dehydrogenase and lactate dehydrogenase in the myelin, synaptosome and mitochondrial fractions prepared from the cerebral cortex homogenates is shown in Figure 2. The identity of the fractions was ascertained from electron micrographs, the preparation and assessment of which were carried out by Dr D.N. Wheatley in the Department of Pathology and the degrees of contamination of fractions were very small. The results illustrate that the synaptosome fraction is enriched in the activities of the enzymes normally associated with nerve terminals, acetylcholinesterase and Na$^+$,K$^+$-ATPase in the membranes and lactate dehydrogenase trapped from the cytoplasm of the cell body, as reported by others (Johnson & Whittaker, 1963; Whittaker, 1959, 1965).

Effects of anticonvulsants and convulsants on synaptosome ATPase activities

None of the drugs tested (Table 1) altered the Mg^{++}-ATPase activity of synaptosomes. Two of the anticonvulsants altered the Na$^+$,K$^+$-ATPase activity, phenytoin (0.2 mM) and ethosuximide (2.5 mM) inhibiting it as we have found previously (Gilbert & Wyllie, 1974a, b; Gilbert, Scott & Wyllie, 1974b). Dose-response curves (for instance Figure 3) for the two anticonvulsants indicated that they inhibited the

Table 1 Effects of drugs on synaptosome ATPase activities

Drug (mM)	*% Inhibition of Na$^+$,K$^+$-ATPase*	P
Phenytoin (0.2)	65.0 ± 9.1 (6)	<0.001
Ethosuximide (2.5)	64.2 ± 5.3 (5)	<0.001
Diazepam (0 25)	56.1 ± 3.8 (4)	<0.01

Values are the mean with s.e. mean and the number of observations is given in parentheses. Phenobarbitone (1.0), acetazolamide (0.1), trimethadione (1.0), dimethadione (1.0), SC-13504 (2 µg/ml), leptazol (1.0), strychnine (0.2), picrotoxin (1.0) and barbitone (1 0) had no effect and none of the drugs tested altered the Mg^{++}-ATPase activity. Typical control values (µmol Pi.mg protein^{-1} h^{-1}) Na$^+$,K$^+$-ATPase 9.70 ± 0.79 (22), Mg^{++}-ATPase 6.60 ± 0.54 (22).

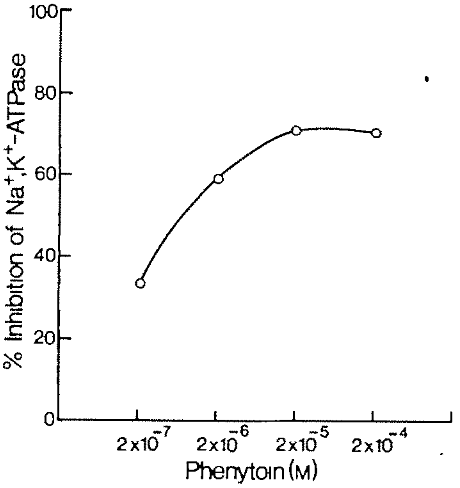


Figure 3 Typical log dose-response plot for the inhibition of synaptosomal Na^+,K^+-ATPase by phenytoin.

activity *in vitro* at concentrations which are similar to those likely to be present *in vivo* during anticonvulsant therapy (Millichap, 1965; Eadie & Tyrer, 1974), that is 0.04 mM for phenytoin and 0.5 mM for ethosuximide. Diazepam (0.25 mM) which has found use in the treatment of status epilepticus (Eadie & Tyrer, 1974) also inhibited the Na^+,K^+-ATPase activity.

Effects of anticonvulsants on the ATPases of synaptosome components

Osmotic disruption of synaptosomes and subsequent separation of the components into fractions containing mainly membranes, synaptic vesicles or mitochondria permitted investigations of the ATPases in these components separately, although a very small degree of contamination of each fraction by material from another was inevitable and was apparent in electron micrographs.

The relative specific activities of Na^+,K^+-ATPase, acetylcholinesterase, succinate dehydrogenase and lactate dehydrogenase in the fractions are shown in Figure 4 and they are in agreement with the results of Whittaker *et al.* (1964) and Hosie (1965) who used similar techniques to isolate components of synaptosomes. Considering Figure 4 in conjunction with Figure 1 it can be seen that the material floating above the least dense sucrose layer (0.4 M), fraction O, contained lactate dehydrogenase activity but little activity of any of the other enzyme markers. These findings are compatible with the proposed cytoplasmic origin of this material (Whittaker, 1965). Fraction D, the material suspended in the 0.4 M sucrose, was shown by electron microscopy to contain the synaptic vesicles. It contained no detectable Na^+,K^+-ATPase activity and very little of the activities associated with the other enzymes. Other studies (see later) have shown that this fraction contains Mg^{++}-ATPase.

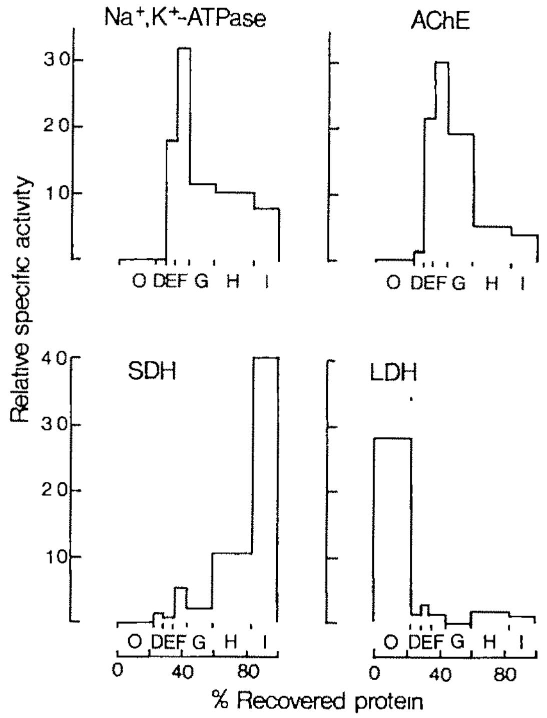


Figure 4 Distribution of the cytoplasmic marker lactate dehydrogenase (LDH), the mitochondrial marker succinate dehydrogenase (SDH) and membrane markers Na^+,K^+-ATPase and acetylcholinesterase (AChE) in fractions (see Figure 1) prepared from disrupted synaptosomes prepared by density gradient centrifugation The ordinates (relative specific activity) are the percentages of enzyme recovered in the fractions divided by the percentages of protein recovered in the same fraction.

The activities of acetylcholinesterase and Na^+,K^+-ATPase, enzymes generally associated with membranes, were high in the material deposited at the interphases between the 0.4–0.6 M, 0.6–0.8 M and 0.8–1.0M sucrose solutions, fractions E, F and G respectively. Electron microscopy suggested that the material in the three fractions was derived from membranes. Comparatively little succinate or lactate dehydrogenase activity was detected in these fractions and since no obvious difference, other than the different densities, was apparent between them the fractions were pooled as the 'membrane' fraction for subsequent studies.

Fraction H (between 1.0 and 1.2 M sucrose) contained ruptured synaptosomes, the various components of the original particles adhering one to another. High acetylcholinesterase, Na$^+$,K$^+$-ATPase and succinate dehydrogenase activities were apparent in this fraction but, as one might expect, very little of the activity of the cytoplasmic enzyme lactate dehydrogenase.

The pellet at the base of the centrifuge tube (fraction I) was composed mainly of mitochondria which had been contained originally in the synaptosomes. The mitochondrial enzyme succinate dehydrogenase showed high activity in the fraction and the activities of acetylcholinesterase and Na$^+$,K$^+$-ATPase were also marked. Very little lactate dehydrogenase activity was present.

Table 2 shows that none of the anticonvulsants tested altered either the Mg^{++}- or the Na$^+$,K$^+$-ATPase activity of the mitochondrial fraction. The lack of an effect of ethosuximide is in agreement with our previous results (Gilbert & Wyllie, 1974a). Ethosuximide (2.5 mM) and phenytoin (0.2 mM) each inhibited the Na$^+$,K$^+$-ATPase of the membrane fraction markedly.

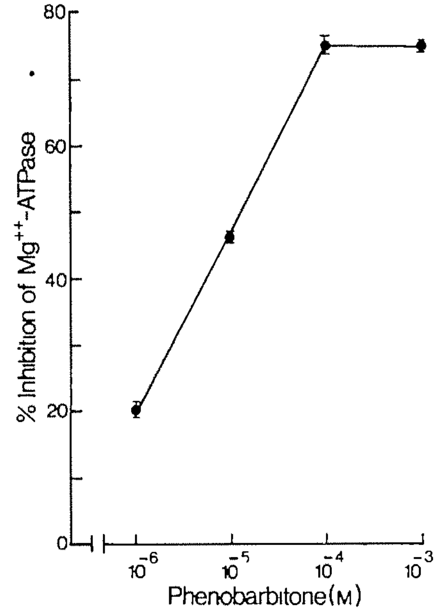


Figure 5 Log dose-response plot for the inhibition of vesicular (D fraction) Mg^{++}-ATPase by phenobarbitone. Vertical bars indicate s.e mean of 3 or 4 experiments.

Experiments with the synaptic vesicles (fraction D) gave particularly interesting results (Table 3). There was no detectable Na$^+$,K$^+$-ATPase activity in the vesicular fraction. Mg^{++}-ATPase was present, however, and its activity was comparable to that of the membrane fraction which was approximately 50% of the activity in the mitochondrial fraction. All of the anticonvulsant drugs inhibited the Mg^{++}-ATPase activity of the vesicles, whereas the Mg^{++}-ATPase activities of the other fractions were not influenced by the drugs. Ouabain, barbitone and the convulsants leptazol, strychnine and picrotoxin did not alter the vesicular Mg^{++}-ATPase activity significantly at the relatively high concentrations indicated, and lower concentrations were also ineffective. Diazepam (0.25 mM) inhibited the enzyme by some 63%. This

Table 2 The effects of anticonvulsants on the ATPase activities of the membrane and mitochondrial fractions prepared by osmotic lysis of synaptosomes

	Membrane fraction ATPase activity (μmol Pi.mg protein^{-1} h^{-1})	
Drug (mM)	*Na$^+$,K$^+$-*	*Mg^{++}-*
Control	11.56 ± 1 19 (33)	5.26 ± 0.56 (31)
Phenytoin (0.2)	2.89 ± 0.79 (9)**	5 30 ± 0.80 (9)
Ethosuximide (2.5)	3.61 ± 0.81 (12)**	4.82 ± 0 61 (12)
Phenobarbitone (1.0)	10.38 ± 2.30 (6)	5.48 ± 1.15 (5)
Acetazolamide (0.1)	9.71 ± 1.78 (7)	4 93 ± 1.39 (5)
	Mitochondrial fraction	
Control	10.40 ± 1.70 (9)	10.32 ± 0.39 (9)
Phenytoin (0.2)	6.82 ± 2.61 (3)	10.80 ± 0.79 (3)
Ethosuximide (2.5)	8.51 ± 3.30 (4)	10.40 ± 1.42 (2)
Phenobarbitone (1.0)	12.98 ± 0.12 (2)	8.87 ± 2.46 (2)
Acetazolamide (0.1)	12.82 ± 0.44 (2)	8.82 ± 2.66 (2)

The number of observations is given in parentheses. Values are the mean with s.e. mean. For tests of significance only paired drug and control results were included in the calculations

** $P < 0.001$.

Table 3 Effects of drugs on the Mg^{++}-ATPase activity of the vesicle-containing fraction

Drug (mM)	*% Inhibition of Mg^{++}-ATPase activity*	P
Control		
Phenytoin (0.2)	54.9 ± 3.9 (3)	<0.001
Ethosuximide (2.5)	73.7 ± 7.0 (4)	<0.001
Phenobarbitone (1.0)	74 9 ± 1.6 (2)	<0.025
Acetazolamide (0.1)	52.8 ± 8.1 (3)	<0.025
Trimethadione (1.0)	70.9 ± 2.8 (3)	<0.005
Dimethadione (1.0)	62.8 ± 6.2 (3)	<0.010
SC 13504 (0 05)	79.5 ± 4.5 (3)	<0.025
Diazepam (0.25)	62.2 ± 7.5 (3)	<0.025

Values are the mean with s.e. mean and the number of observations is given in parentheses Leptazol (1 0), strychnine (0.25), picrotoxin (1.0), barbitone (1 0) and ouabain (1.0) had no effect. Typical control value (μmol Pi.mg $protein^{-1}$ h^{-1}) 5.10 ± 0.31 (16).

was the maximum degree of inhibition shown by diazepam in a dose-response curve.

Inhibition of the Mg^{++}-ATPase by anticonvulsants did not exceed a maximum of approximately 80% of the enzymatic activity in dose-response curves and this is seen, for example, in Figure 5 which shows the curve obtained with phenobarbitone. All the anticonvulsants exerted near maximum effect at concentrations reported to be effective therapeutically.

Discussion

Any attempts to evaluate effects of drugs on nerve terminals isolated from the central nervous system and studied *in vitro* must depend for their validity upon the integrity of the preparation derived from the tissue. With this in mind, certain criteria were selected as indices of the quality of the synaptosomes used in the present work.

Electron microscopy, performed and assessed in another department (see results section) showed that the synaptosome fraction was reasonably homogeneous and largely uncontaminated by free mitochondria. The synaptosomes were between 0.45 and 0.6 μm in diameter and often retained attached membrane material which was presumably postsynaptic in origin. The fraction was enriched in the activity of acetylcholinesterase, an enzyme localized in presynaptic membranes of cholinergic neurones (Lewis & Shute, 1964). The activity of succinate dehydrogenase in the synaptosome fraction was presumably due to the presence of intra-synaptosomal mitochondria. Lactate dehydrogenase, a cytoplasmic enzyme which becomes trapped to some extent in the synaptosome when the nerve terminal ruptures and reseals during the preparative procedure was retained in the synaptosome fraction as reported by others (Johnson & Whittaker, 1963). Na^+,K^+-ATPase, a membrane bound enzyme which has been reported to be more highly localized in the synaptosome fraction, showed a distribution compatible with that reported by other workers who have used similar techniques to isolate the nerve ending particles (Hosie, 1965).

We have assumed, in view of these observations, that the effects of the drugs on the synaptosomes reliably reflect effects on nerve terminals *in vitro* rather than effects on material contaminating the nerve terminal fractions and derived from other, less discrete regions of the cell.

With regard to the synaptosome fraction itself only ethosuximide and phenytoin significantly inhibited Na^+,K^+-ATPase activity whilst none of the drugs influenced Mg^{++}-ATPase activity. Similar results have been obtained previously (Gilbert *et al.*, 1974a, b; Gilbert & Wyllie, 1974a, b). Formby (1970) and Escueta & Appel (1970) have also reported inhibition of cerebral cortex Na^+,K^+-ATPase by phenytoin but Festoff & Appel (1968) found that when the ratio of sodium to potassium ions in the assay medium was above 25 : 1 phenytoin stimulated the enzyme. The present results do not confirm that report but it may be that it is the absolute concentrations of the ions, rather than the relative concentrations which are important.

Just how inhibition of Na^+,K^+-ATPase might be involved in anticonvulsant activity is a matter for conjecture. The reported inhibition of post-tetanic potentiation by phenytoin (Esplin, 1957) could well result from inhibition of the enzyme with the result that sodium ions could accumulate in the presynaptic region during repetitive stimulation. Thus sodium ions would not be actively extruded and no hyperpolarization would occur at a time when this would otherwise be the case due to increased Na^+,K^+-ATPase activity. One result could be depressed neurotransmitter release from the nerve terminal.

Like ethosuximide and phenytoin, diazepam inhibited synaptosome Na^+,K^+-ATPase. This result is in agreement with the work of Davis & Brody (1966) and Ueda, Wada & Ballinger (1971) who suggested that the pharmacological activity of diazepam was mediated through this effect. However, this view is not generally accepted (see for instance Landmark & Øye, 1971) and its relationship to the value of diazepam in the treatment of status epilepticus is obscure.

A possible role of Na^+,K^+-ATPase in the regulation of neurotransmitter release merits discussion here. Paton *et al.* (1971) and Vizi (1972) obtained circumstantial evidence to suggest that acetylcholine release at peripheral and central cholinergic synapses can be triggered by a depression of Na^+,K^+-ATPase activity. In their experiments, conditions which, in other tissues, have been shown to inhibit Na^+,K^+-ATPase activity were found to stimulate acetylcholine release from myenteric plexus and from cerebral cortex slices. Treatment of the myenteric plexus-longitudinal muscle preparation of the guinea-pig ileum with ouabain or *p*-hydroxymercuribenzoate, or alternatively subjecting the tissue to sodium ion depletion, increased acetylcholine release and these conditions were assumed to inhibit Na^+,K^+-ATPase. Although no determinations of the activity of the enzyme were made, there is little reason to doubt the validity of the assumption and it is also reasonable to assume that the enzyme concerned is in the nerve terminal. If acetylcholine release is regulated by this enzyme, then the effects of ethosuximide, phenytoin and diazepam could also cause transmitter release at certain central nervous system cholinergic synapses if the drugs gain access to the necessary sites. Experiments to test this possibility are in progress. However, in considering the possible roles of Na^+,K^+-ATPase in the nerve terminal it should be remembered that anticonvulsants other than ethosuximide and phenytoin did not influence the activity of the enzyme when preparations containing intact synaptosomes were added to the ATPase assay medium. It could be argued that any effect of the anticonvulsants on the membrane ATPase of the synaptosome is masked when the synaptosome bursts to release mitochondria and other components on contact with the hypotonic imidazole/HCl buffer. This emphasizes the importance of the experiments designed to test the effects of the drugs on the membranes, mitochondria and synaptic vesicles, isolated from the synaptosomes and studied separately. The three general components were found to have quite different enzyme characteristics. Na^+,K^+ATPase activity was pronounced in both the membrane and mitochondrial components but, as reported previously (Gilbert & Wyllie, 1974a), only the former was sensitive to ethosuximide. In contrast to the other components the vesicles contained no detectable Na^+,K^+-ATPase activity.

In agreement with the results obtained with synaptosomes, ethosuximide and phenytoin were the only anticonvulsants to alter Na^+,K^+-ATPase activity in any of the subfractions, the effect being to inhibit the enzyme in the membrane fraction.

The most striking finding is that all of the anticonvulsant drugs tested inhibited the Mg^{++}-ATPase activity of the vesicles. The Mg^{++}-ATPase activities of other fractions were not influenced by the drugs and since the activity of the enzyme in the vesicles is similar to that in the synaptosome itself, effects of the drugs on total synaptosome Mg^{++}-ATPase may not be detectable because the vesicles contribute only some 5% to synaptosomal protein. The degree of inhibition of the Mg^{++}-activated enzyme in the vesicles did not exceed 80%, however, and it may be that this reflects a degree of heterogeneity of the fraction, either the 20% of activity which remains is contained in material which contaminates the fraction and which is not vesicular in origin, or this 20% could represent the activity of vesicles of a synaptic type which is different from the type contributing the 80% of activity which is sensitive to the anticonvulsants. This intriguing possibility requires investigation. Clearly, the significance of the Mg^{++}-ATPase has to be established. There is evidence to suggest that a relationship exists between the activity of a Mg^{++}-ATPase and the ability of chromaffin granules of the adrenal medulla to store noradrenaline. Banks (1965) and Kirshner, Smith & Kirshner (1966) showed that the membrane of the storage vesicles contains Mg^{++}-ATPase activity and Taugner & Hasselbach (1968) observed that the degree of inhibition of the enzymes by *N*-ethylmaleimide correlated well with the degree of inhibition of catecholamine uptake into the vesicles. The uptake process appears to be one of active transport, but the pH-dependence of the process suggests that it may not be linked directly to Mg^{++}-ATPase activity (Taugner, 1971; Phillips, 1974). This work does not have a direct bearing upon the relationship between vesicle Mg^{++}-ATPase and transmitter uptake and release in the central nervous system but it does suggest the processes may be related. In this connection the finding that the vesicles exhibit Mg^{++}-ATPase activity but negligible Na^+,K^+-ATPase activity is important in that it will permit studies of the role of the former. In most tissues Mg^{++}-ATPase activity is very closely associated with Na^+,K^+-ATPase activity although it can be separated from it to some extent.

Brief comment should be made concerning inhibition of Mg^{++}-ATPase and anticonvulsant activity. The two phenomena may be unrelated and certainly no relationship was apparent between the clinical uses of the individual anticonvulsants and their ability to inhibit the enzyme. Preliminary studies do suggest, however, that the relative inhibitory potencies of the drugs are of the same order as their potencies with regard to elevation of seizure thresholds in mice and rats. Further experiments are necessary to clarify this point.

This work was supported by a grant from Parke-Davis (to J.C.G.). Acknowledgement is made of generous gifts of the following drugs: dimethadione and trimethadione by Abbot Laboratories, ethosuximide by Parke-Davis and SC-13504 by G.D. Searle. We wish to thank Mrs Helen Anderson for excellent technical assistance.

References

ALDRIDGE, W.N. & JOHNSON, M.K. (1959). Cholinesterase, succinic dehydrogenase, nucleic acids, esterase and glutathione reductase in sub-cellular fractions from rat brain. *Biochem. J.*, **73**, 270–276.

BANKS, P. (1965). The adenosine-triphosphatase activity of adrenal chromaffin granules. *Biochem. J.*, **95**, 490–496.

BONTING, S L, SIMON, K.A. & HAWKINS, N.M (1961). Studies on sodium-potassium-activated adenosine triphosphatase. 1. Quantitative distribution in several tissues of the cat. *Archs Biochem. Biophys.*, **95**, 416–423.

DAVIS, P.W. & BRODY, T M (1966). Inhibition of Na^+,K^+-activated adenosine triphosphatase in rat brain by substituted phenothiazines. *Biochem. Pharmac.*, **15**, 703–710.

EADIE, M J. & TYRER, J.H. (1974). *Anticonvulsant therapy: pharmacological basis and practice*, pp. 125–135. Edinburgh: Churchill Livingstone.

ESCUETA, A.V. & APPEL, S.H (1970). The effects of electrically induced seizures on potassium transport within isolated nerve terminals. *Neurology, Minneap.*, **20**, 392.

ESPLIN, D.W. (1957). Effects of diphenylhydantoin on synaptic transmission in cat spinal cord and stellate ganglion. *J. Pharmac. exp. Ther.*, **120**, 301–323.

FESTOFF, B.W. & APPEL, S.H. (1968). Effect of phenytoin on synaptosome sodium-potassium-ATPase. *J. clin. Invest.*, **47**, 2752–2758.

FORMBY, B (1970). The *in vivo* and *in vitro* effect of diphenylhydantoin and phenobarbitone on K^+-activated phosphohydrolase and (Na^+,K^+)-activated ATPase in particulate membrane fractions from rat brain. *J. Pharm. Pharmac.*, **22**, 81–85.

GILBERT, J.C., BUCHAN, P. & SCOTT, A.K. (1974a). Effects of anticonvulsant drugs on monosaccharide transport and membrane ATPase activities of cerebral cortex. *Hans Berger symposium on epilepsy*, pp. 98–104. Edinburgh: Churchill.

GILBERT, J.C., GRAY, P. & HEATON, G.W. (1971). Anticonvulsant drugs and brain glucose. *Biochem. Pharmac.*, **20**, 240–243.

GILBERT, J.C, SCOTT, A K & WYLLIE, M.G. (1974b). Effects of ethosuximide on adenosine triphosphatase activities of some subcellular fractions prepared from rat cerebral cortex. *Br. J. Pharmac.*, **50**, 452–453P.

GILBERT, J.C. & WYLLIE, M.G. (1974a). The effects of the anticonvulsant ethosuximide on adenosine triphosphatase activities of synaptosomes prepared from rat cerebral cortex. *Br. J. Pharmac.*, **52**, 139–140P.

GILBERT, J.C. & WYLLIE, M G (1974b). The effects of phenytoin on adenosine triphosphatase activities of synaptosomes and their components. *Br. J. Pharmac.*, **52**, 445P.

GILBERT, J C & WYLLIE, M.G. (1975). Effects of prostaglandins on the ATPase activities of synaptosomes. *Biochem. Pharmac.*, **24**, 551–556.

GILBERT, J.C., WYLLIE, M.G. & DAVISON, D.V. (1975). Nerve terminal ATPase as possible trigger for neurotransmitter release. *Nature, Lond.*, **255**, 237–238.

GRAY, E.G. & WHITTAKER, V.P. (1962). The isolation of nerve endings from brain: an electron microscope study of cell fragments derived by homogenization and centrifugation. *J. Anat.*, **96**, 79–87.

HOSIE, R.J.A. (1965). The localization of adenosine triphosphatase in morphologically characterised fractions of guinea-pig brain. *Biochem. J.*, **96**, 404–412.

JOHNSON, M K (1960). The intracellular distribution of glycolytic and other enzymes in rat brain homogenates and mitochondrial preparations. *Biochem. J.*, **77**, 610–618.

JOHNSON, M.K & WHITTAKER, V.P. (1963). Lactate dehydrogenase as a cytoplasmic marker in brain. *Biochem. J.*, **88**, 404–409.

KIRSHNER, N., SMITH, W.J. & KIRSHNER, A.G. (1966). Uptake and storage of catecholamines. In *Mechanisms of release of biogenic amines*, ed. Von Euler, U.W., Rosell, S. & Unväs, B., **5**, 109–123.

KOOSTRA, A. & WOODHOUSE, S.P. (1974). The effect of diphenylhydantoin on Na^+,K^+-stimulated ouabain inhibited ATPase. *Proc. Univ. Otago Med. Sch.*, **52**, 6–7.

LANDMARK, K. & ØYE, I. (1971). The action of thioridazine and promazine on biological membranes: relationship between ATPase inhibition and membrane stabilization. *Acta pharmac. tox.*, **29**, 1–8.

LEWIS, P.R. & SHUTE, C C.D (1964). Demonstration of cholinesterase activity with the electron microscope. *J. Physiol.*, **175**, 5–7P.

LOWRY, O H., ROSENBROUGH, N., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

MILLICHAP, J.G. (1965). Anticonvulsant drugs. *Physiological pharmacology* II, pp. 97–173. New York and London: Academic Press.

NAHORSKI, S.R. (1972). Biochemical effects of the anticonvulsants trimethadione, ethosuximide and chlordiazepoxide in rat brain. *J. Neurochem.*, **19**, 1937–1946.

PATON, W D.M, VIZI, E.S. & ZAR, M.A (1971). The mechanism of acetylcholine release from parasympathetic nerves. *J. Physiol.*, **215**, 819–848.

PHILLIPS, J.H. (1974). Steady-state kinetics of catecholamine transport by chromaffin granule 'ghosts'. *Biochem. J.*, **144**, 319–325.

QUAESTEL, J.H. & WHEATLEY, A.H.M. (1938). On Ferricyanide as a reagent for the manometric investigation of dehydrogenase systems. *Biochem. J.*, **32**, 936–943.

RAWSON, M.D. & PINCUS, J H. (1968). The effect of diphenylhydantoin on sodium, potassium, magnesium-activated adenosine triphosphatase in microsomal fractions of rat and guinea-pig brain and whole homogenates of human brain. *Biochem. Pharmac.*, **17**, 573–581.

TAUGNER, G. (1971). The membrane of catecholamine storage vesicles of adrenal medulla. Catecholamine fluxes and ATPase activity. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **270**, 392–406.

TAUGNER, G & HASSELBACH, V.V. (1968). Die Bedeutung der Sulfhydryl-Gruppen für den Catecholamin Transport der Vesikel des Nebennierenmarkes. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **260**, 58–79.

UEDA, I, WADA, T. & BALLINGER, C.M. (1971). Sodium- and potassium-activated ATPase of some tranquillizers. *Biochem. Pharmac.*, **20**, 1697–1700.

VIZI, E.S. (1972). Stimulation of acetylcholine release from cortical slices of rat brain by inhibition of Na^+ K^+ Mg^{++}-

ATPase. *J. Physiol.*, **226**, 95–117.

WHITTAKER, V.P. (1959). The isolation and characterization of acetylcholine-containing particles from rat brain. *Biochem. J.*, **72**, 694–706.

WHITTAKER, V.P. (1965). The application of subcellular fractionation techniques to the study of brain function. *Prog. Biophys. Mol. Biol.*, **15**, 39–96.

WHITTAKER, V.P., MICHAELSON, I.A. & KIRKLAND, R.J.A. (1964). The separation of synaptic vesicles from nerve-ending particles ('Synaptosomes'). *Biochem. J.*, **90**, 293–303.

WOODBURY, D.M. & KEMP, J.W. (1970). Some possible mechanisms of action of antiepileptic drugs. *Pharmakopsychiatrie Neuro-Psychopharmakol.*, **3**, 201–226.

(Received May 29, 1975.
Revised August 20, 1975.)

Br. J. Pharmac. (1976), **56**, 59–68

STIMULANT PROPERTIES OF BROMOCRIPTINE ON CENTRAL DOPAMINE RECEPTORS IN COMPARISON TO APOMORPHINE, (+)-AMPHETAMINE AND L-DOPA

A.M. JOHNSON[1], D.M. LOEW & J.M. VIGOURET
Medical and Biological Research Division, SANDOZ LTD., Basle, Switzerland

1 The activity of bromocriptine has been investigated in tests for the stimulation of central dopaminergic mechanisms. The results obtained have been compared with those of apomorphine, (+)-amphetamine and L-DOPA.

2 Bromocriptine (2.5 to 10 mg/kg) induced stereotyped sniffing and licking in rats. The stereotypy was more intense than that induced by L-DOPA and less intense than that of apomorphine and (+)-amphetamine over the dose ranges studied.

3 In rats lesioned unilaterally in the substantia nigra by local injection of 6-hydroxydopamine, bromocriptine, like apomorphine and L-DOPA, induced turning contralateral to the side of the lesion. The smallest dose of bromocriptine to induce turning was 0.5 mg/kg.

4 Reserpine-induced catalepsy in mice was antagonized by bromocriptine, with an ED_{50} of 1.8 mg/kg. It was intermediate in potency to apomorphine and L-DOPA.

5 Spontaneous locomotor activity in mice was stimulated by bromocriptine in a dose-dependent manner from 2.5 to 10 mg/kg after an initial suppression of activity.

6 In all experiments, bromocriptine was characterized by a prolonged duration of activity after a delay in the onset of effect.

7 The stereotyped behaviour induced by bromocriptine was inhibited by prior administration of pimozide, reserpine or α-methyl-*p*-tyrosine.

8 Bromocriptine-induced turning behaviour was abolished by pretreatment with pimozide, and reduced after α-methyl-*p*-tyrosine pretreatment.

9 The results obtained support the conclusion that bromocriptine acts by stimulating dopamine receptors in the central nervous system and that intact catecholamine synthesis and granular amine storage mechanisms are necessary for it to bring about its effects.

Introduction

Bromocriptine (CB 154), 2-bromo-α-ergocryptine (Figure 1) is an ergot polypeptide derivative comprising a lysergic acid residue with a cyclic polypeptide moiety.

It was originally characterized as a specific inhibitor of prolactin secretion, being active in all vertebrates tested so far, including man (Flückiger, 1972; Del Pozo, Brun Del Re, Vorga & Fiesen, 1972).

Preliminary investigations into the mechanism of action of bromocriptine indicated that it acted directly on prolactin secreting cells of the hypophysis (Marko & Niederer, unpublished results; Pasteels, Danguy, Frerotte & Ectors, 1971). However, Hökfelt & Fuxe (1972) found that, in the rat, bromocriptine also decreased dopamine (DA) turnover in the median eminence and suggested that it might interfere with dopaminergic tuberoinfundibular neurones which allegedly control prolactin secretion. During the course of investigations into the role of these neurones, using bromocriptine as a tool to inhibit prolactin secretion, Corrodi, Fuxe, Hökfelt, Lidbrink & Ungerstedt (1973) discovered that it also decreased DA turnover in the neostriatum, an action attributable to a direct stimulation of DA receptors at this site. This central dopaminergic stimulation is assumed to be responsible for the significant therapeutic action of bromocriptine in patients with idiopathic Parkinsonism (Calne, Leigh, Teychenne, Bamji &

---

[1] Present address: Medicinal Research Centre, Beecham Pharmaceuticals, The Pinnacles, Fourth Avenue, Harlow, Essex.

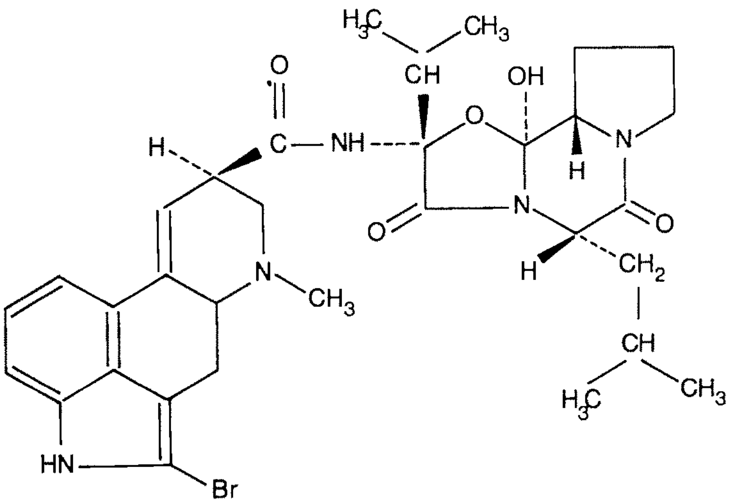


Figure 1 Structure of bromocriptine (2-bromo-α-ergocryptine, CB 154)

Greenacre, 1974a; Calne, Teychenne, Claveria, Eastman, Greenacre & Petrie, 1974b). We have now investigated the activity of bromocriptine in tests sensitive to dopaminergic mechanisms in the central nervous system. Bromocriptine has been compared to L-DOPA, apomorphine and (+)-amphetamine because of their established actions on central dopaminergic mechanisms.

Some preliminary experiments with bromocriptine in comparison to other ergotoxine alkaloids have already been reported (Johnson, Vigouret & Loew, 1973, 1974).

Methods

Production of stereotyped behaviour in rats

Rats, 180–220 g, were placed in perspex cylinders of 30 cm diameter on a wire grid floor. After 30 min acclimatization to the cage, the rats were injected with the compound under investigation. The behaviour of the rats was observed for 2 min at 30 min intervals for 2 h and then at 60 min intervals for a total of up to 7 hours. The degree of stereotyped behaviour observed was assessed using a scoring system based on that described by Costall, Naylor & Olley (1972).

The scores and criteria are as follows: 1 = intermittent sniffing; 2 = persistent sniffing, occasional licking; 3 = licking, occasional biting; 4 = intense and persistent biting. The ratings were carried out by an observer who was unaware of the treatment the rats received. Six rats were investigated at each dose level.

The induction of turning in rats lesioned unilaterally in the substantia nigra by 6-hydroxydopamine

Rats were lesioned by the method described by Ungerstedt (1971), using rats weighing 145–155 g when lesioned.

Four μl of a solution, containing 2 mg/ml of 6-hydroxydopamine hydrochloride, calculated as base, in 0.9% NaCl solution with 0.2 mg/ml ascorbic acid as antioxidant, was slowly injected unilaterally (right) into the medial substantia nigra of rats under sodium pentobarbitone anaesthesia.

One week later, the rats were challenged with 0.25 mg/kg s.c. apomorphine. Rats responding by turning contralaterally to the side of the lesion, i.e. to the left, at not less than 5 turns/min were selected for further experiments. All experiments were carried out in rats that had been lesioned at least 4 weeks previously. After injection, the number of turns occurring in 2 min every 30 min in the case of bromocriptine or every 10 min for other drugs was counted. The direction of the turns was recorded in all instances. Rats were observed until turning stopped or up to 9 h after injection. The total number of turns produced by each treatment was also calculated.

In a preliminary experiment with a group of 6 rats, single doses of bromocriptine (2 mg/kg), apomorphine (0.5 mg/kg), (+)-amphetamine (5 mg/kg) and L-DOPA (100 mg/kg) were investigated. The activity of L-DOPA (25 mg/kg) alone and after pretreatment of the rats with the peripheral DOPA decarboxylase inhibitor, Ro 4-4602, was also tested.

In a second experiment, the effects of 3 doses of apomorphine and bromocriptine were compared in another group of 6 rats. Treatments were allocated to the rats by means of a Latin square design. The experiments were carried out on alternate days over a period of 2 weeks. Differences between treatments were analysed by means of Student's *t*-test for paired observations.

Antagonism of reserpine-induced catalepsy in mice

Mice (18–25 g) in groups of 10 were injected intraperitoneally with reserpine (5 mg/kg) 17 h before subcutaneous administration of the compounds under investigation. Catalepsy was assessed at 30 min, 1 h, 2 h, 3.5 h and, in the case of bromocriptine, 5 h after administration of the compound using the method of Zetler & Moog (1958). The number of mice showing antagonism of catalepsy, i.e. with the ability to walk off a twine-covered vertical pole in a co-ordinated manner, was recorded. Control animals retain their position when placed on the side of the pole.

The percentage antagonism of catalepsy in the treated groups compared to control was calculated. At least 3 doses of each compound were investigated and the ED_{50} determined graphically at each observation time after compound administration. The standard error of the ED_{50} was determined by the method of Miller & Tainter (1944).

Stimulation of spontaneous locomotor activity in mice

Spontaneous locomotor activity was recorded by means of a conventional light beam cage. The light and sound proof cabinet contained 4 identical cages, each 22 cm × 37 cm, crossed by 6 light beams. Five mice (18–25 g), were placed in each cage 15 min after compound administration and the number of light beam interruptions taking place was recorded at 30 min intervals for 7 hours. Experiments were repeated 6 times, giving a total of 30 mice per treatment. The mean total motility count for 5 mice over the recording period, and the mean, and s.e. mean, of each 30 min count were calculated. Statistical comparisons were carried out by means of Student's *t*-test.

Drug interaction studies

The effects of pimozide, α-methyl-*p*-tyrosine, and reserpine on the stereotyped and turning behaviour in rats were investigated using the method described previously. The schedules of drug treatment are shown in the table and figures.

Materials and drugs

The drugs used were as follows: bromocriptine mesylate (SANDOZ), L-DOPA (Fluka), (+)-amphetamine sulphate (Merck), apomorphine hydrochloride (SANDOZ), Ro 4-4602 (Hoffmann-La Roche), reserpine, α-methyl-*p*-tyrosine methyl ester (Fluka) and pimozide (Janssen). All doses refer to the available form. In all experiments drugs were administered subcutaneously unless otherwise stated.

SANDOZ-bred male OFA-strain rats and male NMRI mice were used.

Results

Production of stereotyped behaviour in rats

Bromocriptine induced dose-dependent stereotyped behaviour over the dose range 1 to 10 mg/kg. The behaviour was of only moderate intensity, with a peak score of 1.4 at 10 mg/kg, and consisted mainly of sniffing. The activity was slow in onset but was more persistent than that of the other treatments. Apomorphine induced dose-dependent stereotyped behaviour of short duration over the dose range, 0.3 to 10 mg/kg. Biting or gnawing (score 3–4) was observed at 10 mg/kg (Figure 2). (+)-Amphetamine induced a stereotyped behaviour over the dose range 1.25 to 10 mg/kg that was intermediate in intensity and duration of action to that of bromocriptin and apomorphine. The administration of L-DOPA (200 mg/kg) produced only slight changes in the behaviour of the rats. However, after pretreatment of the rats with 50 mg/kg of Ro 4-4602, L-DOPA (200 mg/kg) induced stereotyped behaviour of similar intensity to that of apomorphine (2.5 mg/kg).

Induction of turning in rats lesioned unilaterally in the substantia nigra by injection of 6-hydroxydopamine

Apomorphine (0.5 mg/kg), bromocriptine (2 mg/kg), and L-DOPA (25 mg/kg), the latter with and without pretreatment with Ro 4-4602, induced contralateral turning in the lesioned rats (Figure 3). The total number of turns induced by L-DOPA was dose-dependent and pretreatment of the rats with Ro 4-4602 (50 mg/kg i.p.) caused a significant potentiation in the response to L-DOPA (25 mg/kg). In contrast, (+)-amphetamine (5 mg/kg) induced ipsilateral turning. Treatment with drug vehicles resulted in only a small equal total number of turns to both sides. At the doses used, the total number of turns induced by bromocriptine was greater than that produced by any of the other treatments. This was due to the long duration of activity rather than the maximal rate of turning induced, as is clearly illustrated by the results obtained in the second experiment. None of the other treatments induced turning for longer than 4 hours.

In the second experiment, apomorphine induced dose-dependent turning with onset of activity within 10 min of injection. The total number of turns induced

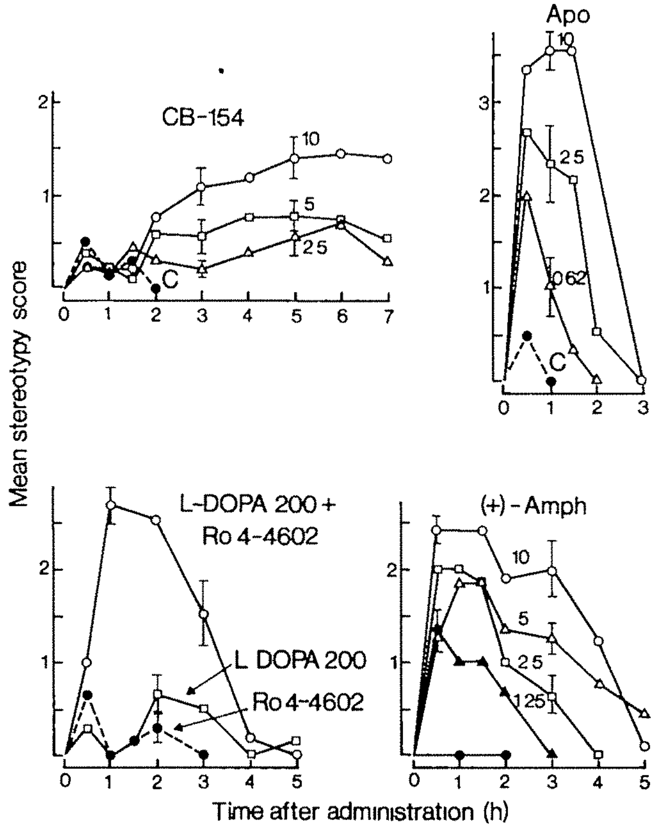


Figure 2 The production of stereotyped behaviour in rats by bromocriptine (CB-154), apomorphine (Apo), L-DOPA before and after intra-peritoneal pretreatment of Ro 4-4602, and by (+)-amphetamine ((+)-Amph). Each point represents the mean sterotypy score of 6 rats Vertical bars are s.e. mean. The figures on the curve indicate the dose administered in mg/kg (C) treatment with drug vehicle.

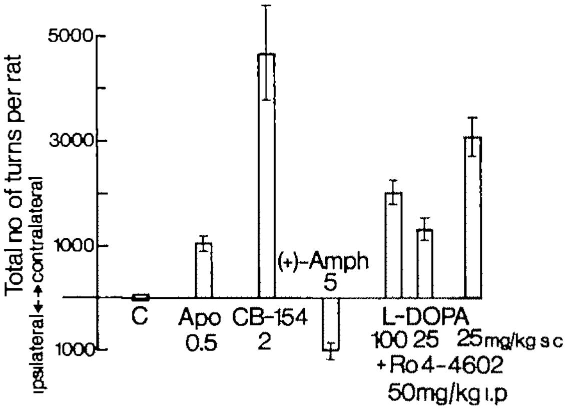
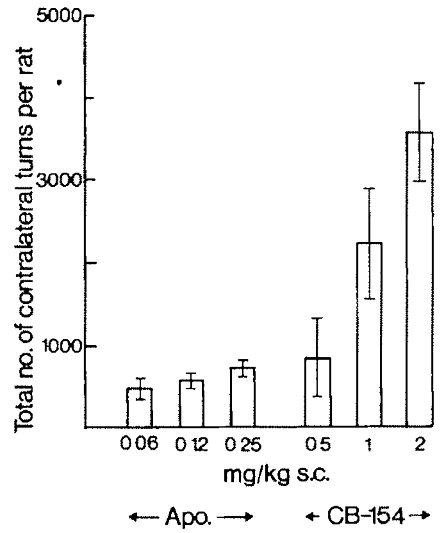


Figure 3 The induction of turning behaviour in rats lesioned unilaterally in the substantia nigra by a local injection of 6-hydroxydopamine after administration of apomorphine (Apo), bromocriptine (CB-154), (+)-amphetamine ((+)-Amph), and L-DOPA, the latter before and after treatment with Ro 4-4602. Each column represents the mean total number of turns induced by each treatment. The vertical bars indicate the s e mean. All treatments were carried out in the same group of 6 rats.

Figure 4 The total number of contralateral turns induced by administration of bromocriptine (CB-154) 0.5, 1 and 2 mg/kg and apomorphine (Apo.) 0.06, 0.12 and 0.25 mg/kg to rats lesioned unilaterally in the substantia nigra by local injection of 6-hydroxydopamine. Vertical bars are the s.e. mean. Turns were recorded for up to a maximum of 9 h after drug administration. A significant difference ($P<0.05$) between the number of turns induced by each of the doses of apomorphine was observed. The difference between the number of turns induced by 0.5 and 2 mg/kg bromocriptine was statistically significant ($P<0.05$).



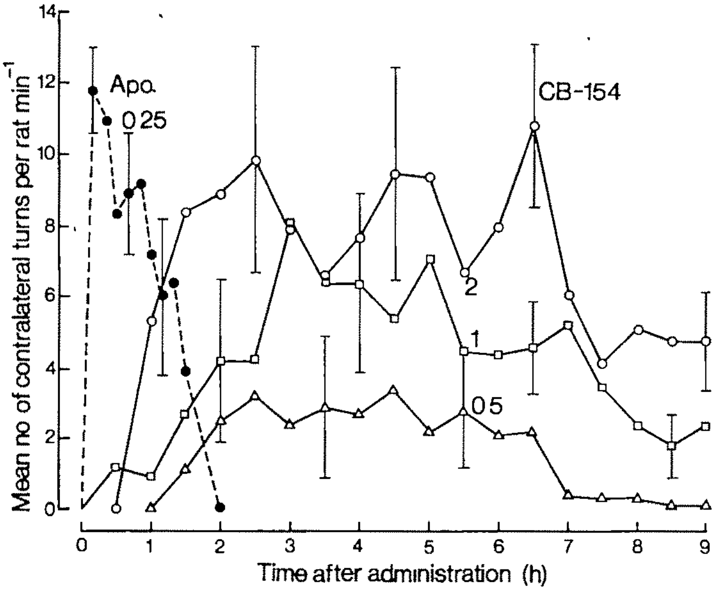


Figure 5 The time course of the induction of turning behaviour in rats lesioned unilaterally in the substantia nigra by a local injection of 6-hydroxydopamine after the administration of bromocriptine (CB-154) 0.5, 1 and 2 mg/kg and apomorphine (Apo.) 0.25 mg/kg. Vertical bars are s.e. mean. The figures on the curve indicate the dose administered in mg/kg. All treatments were administered to the same group of 6 rats. Results obtained in this experiment with apomorphine (0.06 and 0.12 mg/kg) have been omitted for clarity

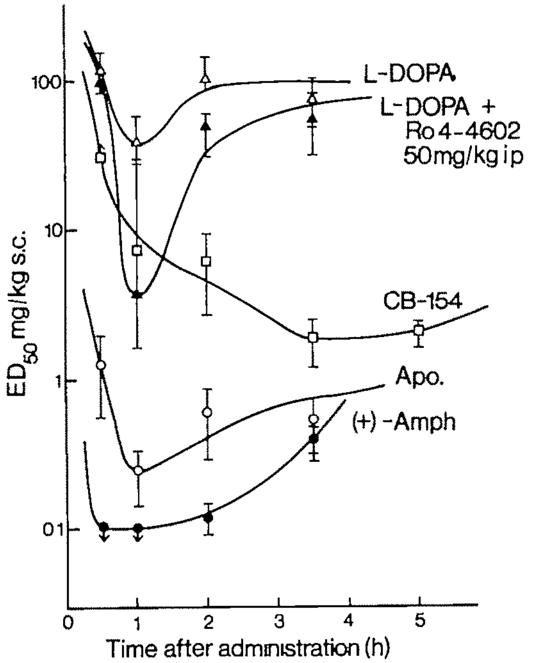


Figure 6 The antagonism of reserpine-induced catalepsy in mice by (+)-amphetamine (+-Amph), apomorphine (Apo), bromocriptine (CB-154) and L-DOPA before and after pretreatment with Ro 4-4602 Results are expressed as the ED_{50} determined at various times after administration of the drugs under investigation. Reserpine (5 mg/kg) was administered by intraperitoneal injection 17 h prior to drug administration. Ten mice per group were used Vertical bars are s.e mean.

by a dose of 0.12 mg/kg was significantly ($P < 0.05$) greater than that induced by 0.06 mg/kg and similarly 0.25 mg/kg induced significantly ($P < 0.05$) more turns than 0.12 mg/kg (Figure 4). Maximal rate of turning by 0.25 mg/kg was observed 10 min after injection (Figure 5).

The effects of bromocriptine were dose-dependent with regard to the total number of turns and maximal rate of turning induced. The differences between the number of turns induced by 0.5 and 1 mg/kg, as well as between 1 and 2 mg/kg were not statistically significant; however, the difference between 0.5 mg/kg and 2 mg/kg was significant ($P < 0.05$; Figure 4). Bromocriptine was characterized by a delay in the onset of activity, but turning was still present at 8 h after 1 and 2 mg/kg and from 2 to 6 h after 0.5 mg/kg. In contrast, turning induced by apomorphine (0.25 mg/kg) had ceased 2 h after injection (Figure 5).

Antagonism of reserpine-induced catalepsy in mice

(+)-Amphetamine was the most potent compound tested and had the most rapid onset of action; ED_{50}'s of less than 0.1 mg/kg were recorded at 30 and 60 min after injection (Figure 6). Apomorphine also showed marked activity, a peak ED_{50} of 0.75 mg/kg was recorded 1 h after injection (Figure 6). Bromocriptine was intermediate in potency to apomorphine and L-DOPA. Its maximum activity occurred 3.5 h after administration with an ED_{50} of 1.8 mg/kg. Bromocriptine differed from all the other compounds tested in that its activity was slow in onset and was well maintained.

L-DOPA alone showed only weak activity against reserpine catalepsy (ED_{50} = 38 mg/kg). However, treatment with the peripheral dopa-decarboxylase inhibitor, Ro 4-4602, 1 h before injection of L-DOPA, potentiated its activity, and a peak ED_{50} of 3.7 mg/kg was recorded 1 h after injection.

Stimulation of spontaneous locomotor activity in mice

Bromocriptine caused a dose-dependent stimulation of locomotor activity in doses of 2.5 to 10 mg/kg (Figure 7) after suppression of the initial exploratory phase. Maximum stimulation was observed 3 to 4 h after drug administration and activity after 10 mg/kg was maintained for 7 h after treatment. Apomorphine (10 mg/kg) only weakly stimulated activity in the second 30 min registration period, and the activity was not maintained. The effect was not statistically significant ($P > 0.05$). (+)-Amphetamine stimulated locomotor activity in dose from 1.25 mg/kg in a dose-dependent manner. Increase in activity was observed even in the initial exploratory phase and lasted over 3 hours. L-DOPA (150 mg/kg) both alone and after pretreatment with Ro 4-4602, significantly stimulated locomotor activity after depressing the initial phase. Pretreatment with Ro 4-4602 produced a greater total response although the peak effect was reduced.

Drug interaction studies

The stereotyped behaviour induced by bromocriptine (10 mg/kg) over 7 h after injection was completely blocked by prior administration of pimozide (1 mg/kg). Similarly α-methyl-*p*-tyrosine (200 mg/kg, 60 min previously) and reserpine (5 mg/kg, 16 h previously), abolished the stereotyped behaviour induced by bromocriptine (10 mg/kg; Table 1). In lesioned rats, turning elicited by bromocriptine (20 mg/kg) was abolished by pimozide, and considerably reduced by previous treatment with α-methyl-*p*-tyrosine (Table 2).

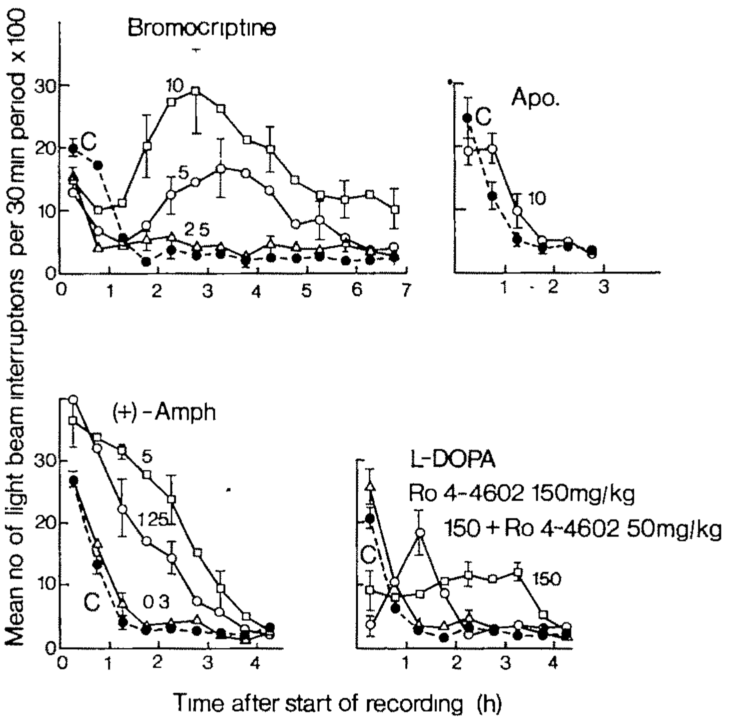


Figure 7 The effect of bromocriptine, apomorphine (Apo), (+)-amphetamine ((+)-Amph) and L-DOPA before and after pretreatment with Ro 4-4602 on the spontaneous locomotor activity of mice. Results are expressed as the mean number of light beam interruptions of a group of 5 mice in 30 min periods. The figures on the curves indicate the dose administered in mg/kg. (C) indicates the effect of vehicle only. Vertical bars are s e. mean. Six groups of 5 mice were investigated for each treatment and dose level. Experiments commenced 15 min after drug administration.

Table 1 Effect of pimozide, α-methyl-*p*-tyrosine and reserpine pretreatment on bromocriptine-induced stereotyped behaviour in the rat

Group	*Treatment*	*Maximal stereotypy score observed*	*P compared with* (a)
(a)	Bromocriptine	1.4	—
(b)	Bromocriptine + pimozide	0	<0 05
(c)	Bromocriptine + AMPT	0	<0.05
(d)	Bromocriptine + reserpine	0	<0.05

Pimozide (1 mg/kg), α-methyl-*p*-tyrosine (AMPT; 200 mg/kg) and reserpine (5 mg/kg) were administered intraperitoneally 1 h, 1 h and 16 h before bromocriptine, respectively The maximal stereotypy score for each rat during 7 h observation following the administration of bromocriptine was recorded. Each value is the mean of 6 rats. Statistical analysis by χ^2 test

Table 2 Effect of pimozide and α-methyl-p-tyrosine on bromocriptine-induced rotation in the lesioned rats

Group	*Treatment*	*Total number of turns* ± *s.e mean*	*P compared with* (a)
(a)	Bromocriptine	2150±640	—
(b)	Bromocriptine + pimozide	0	<0.05
(c)	Bromocriptine + AMPT	694±502	<0.05

Pimozide (1 mg/kg) and α-methyl-p-tyrosine (AMPT; 200 mg/kg) were administered 1 h before bromocriptine. Each value is the mean of 6 rats. Statistical analysis was carried out using Student's *t*-test.

Discussion

It is well established that administration of apomorphine and (+)-amphetamine to rats leads to the production of stereotyped sniffing, licking and biting behaviour (Quinton & Halliwell, 1963; Ernst, 1967; Randrup & Munkvad, 1968), probably due to the stimulation of dopaminergic mechanisms in the striatum (Ernst & Smelik, 1966; Fog, Randrup & Pakkenberg, 1967). The results reported here show that bromocriptine can also induce such behaviour and it may therefore be tentatively concluded that it too activates dopaminergic mechanisms at this site. The stereotyped behaviour induced by bromocriptine was not as intense as that produced by apomorphine or amphetamine; the dose-response curve of bromocriptine was flatter but, after a delay in the onset of action, its duration of action was considerably longer.

The induction of turning by drugs administered to rats with unilateral functional degeneration of the nigro-striatal pathway after the injection of 6-hydroxydopamine into the substantia nigra, has been explained by the suggestion that denervation hypersensitivity develops at striatal DA receptors on the lesioned side (Ungerstedt, 1971). Drugs that mimic the action of DA, e.g. apomorphine (Ernst, 1967; Anden, Rubensson, Fuxe & Hökfelt, 1967), therefore cause a greater stimulation on that side and the rats rotate contralateral to the lesion. Drugs that release DA from neurones, e.g. amphetamine (Ernst, 1967; Fuxe & Ungerstedt, 1968), can do so only on the intact side, causing turning towards this side (ipsilateral turning). Bromocriptine induced turning contralateral to the lesion, indicating that it probably exerts a direct effect at dopamine receptors. These results correspond with those reported by Corrodi *et al.* (1973). The direction of turning observed after administration of apomorphine, (+)-amphetamine and L-DOPA is also in accordance with results reported by Ungerstedt (1971).

The production of catalepsy in animals by neuroleptic drugs is believed to be dependent upon the inhibition of dopaminergic function (van Rossum, Janssen, Boissier, Julou, Loew, Møller-Nielsen, Munkvad, Randrup, Stille & Tedeschi, 1970). Conversely, the neuroleptic-induced cataleptic state is antagonized by administration of drugs known to activate dopaminergic functions, e.g. amphetamine (Morpurgo & Theobald, 1964). In addition, Carlsson, Lindqvist & Magnusson (1957) have reported the antagonism of the tranquillizing action of reserpine by L-DOPA. In the results reported here we have shown that bromocriptine, as well as apomorphine, amphetamine and L-DOPA can antagonize reserpine-induced catalepsy in mice, thus supporting the claim of dopaminergic stimulant properties for this compound. The marked activity of (+)-amphetamine in this test is probably due to the increased sensitivity of reserpinized animals to this compound as reported by Smith (1963), and Morpurgo & Theobald (1966).

Amphetamine and bromocriptine have both been shown to produce dose-dependent stimulation of locomotor activity in mice. In single dose studies, L-DOPA was also effective, the response being modified when mice were pretreated with a dopa-decarboxylase inhibitor. Apomorphine produced only marginal changes in the experimental conditions employed. The stimulation of locomotor activity by amphetamine has been attributed to an action mediated by brain dopamine (Costa, Groppetti & Naimzada, 1972; van Rossum & Hurkmans, 1964; van Rossum, 1970), although noradrenergic mechanism have also been implicated in the action of this class of drugs (Svensson & Waldeck, 1970; Maj, Grobowska & Gajda, 1972; Anden, Strömbom & Svensson, 1973). It is unlikely that noradrenaline is involved in the locomotor stimulant properties of bromocriptin as no pharmacological evidence of the stimulation or facilitation of noradrenaline-dependent processes has yet been obtained (Corrodi *et al.*, 1973). Some reserpine-like activity, i.e. a lowering of cerebral noradrenaline levels has been reported, but this latter effect is more likely to be associated with depression rather than stimulation of spontaneous locomotor activity. Bromocriptine has been shown to be active in four pharmacological models in which activation of central dopaminergic mechanisms have been implicated and in which drugs known to influence these mechanisms have also been shown to be active.

No attempt was made to rank the potencies of the compounds investigated because the methods used to reveal activity differ. Stereotypy was rated in intact rats but in the other methods normal cerebral function had been modified either by a lesion or by systemic drug administration. In these tests, the potency of a compound will not only depend upon the principal pharmacological effect but also on whether it exerts a direct or indirect action on the mechanisms being investigated. In two tests, mice were used and therefore species differences must also be considered. It may be stated, however, that bromocriptine is at least 4 times less active than apomorphine but more potent than L-DOPA. In all tests it possessed a long duration of action, a property that, if confirmed in clinical investigations, would be a considerable advantage over existing therapy for Parkinson's disease.

Further evidence that the effects of bromocriptine are mediated by stimulation of dopamine receptors is that the induction of stereotyped behaviour and contralateral turning was inhibited by pimozide, a dopamine receptor blocking agent (Anden, Butcher, Corrodi, Fuxe & Ungerstedt, 1970). Corrodi *et al.*, 1973, have also reported that the turning induced by bromocriptine in rats lesioned by the method of Ungerstedt (1971), is inhibited by prior administration of pimozide, as is the stimulation of locomotor activity by bromocriptine in mice (Johnson, Loew & Vigouret, in preparation).

The direction of the turning induced by bromocriptine in the lesioned rats suggests that, like apomorphine, it exerts a direct effect on dopamine receptors. However, when animals were pretreated with α-methyl-p-tyrosine, an inhibitor of tyrosine hydroxylase (Spector, Sjoerdsma & Udenfriend, 1965), the turning behaviour and stereotypy are inhibited. This suggests that effects of bromocriptine are dependent upon intact catecholamine synthesis. Similar results have been reported for amphetamine (Weissman, Koe & Tenen, 1966; Hanson, 1966; Dingell, Owens, Norwich & Sulser, 1967; Dominic & Moore, 1969). However, the stereotypy induced by bromocriptine was inhibited in rats pretreated with reserpine, whereas the activity of amphetamine has been reported to be potentiated by this pretreatment (Quinton & Halliwell, 1963; Stolk & Rech, 1968; Dominic & Moore, 1969), and that of apomorphine is not influenced (Ernst, 1967; Maj *et al.*, 1972).

As reserpine blocks the uptake mechanisms in amine storage granules (Carlsson, Hillarp & Waldeck, 1963), bromocriptine appears to require this intact granular amine storage in order to bring about its effects, thus contrasting with both (+)-amphetamine and apomorphine in its mode of action. The inhibition of the bromocriptine-induced locomotor stimulation in mice by prior administration of either α-methyl-p-tyrosine or reserpine has also recently been observed (Johnson, Vigouret & Loew, unpublished).

In conclusion, therefore, it may be stated that bromocriptine stimulates central dopamine receptors and differs in its mode of action from both amphetamine and apomorphine.

References

ANDEN, N.-E., BUTCHER, S.G , CORRODI, H., FUXE, K. & UNGERSTEDT, U. (1970). Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *Eur. J. Pharmac.*, **11**, 303–314.

ANDEN, N.-E., RUBENSSON, E., FUXE, K. & HÖKFELT, T. (1967). Evidence for dopamine receptor stimulation by apomorphine. *J. Pharm. Pharmac.*, **19**, 627–629.

ANDEN, N.-E., STRÖMBOM, U & SVENSSON, T.H. (1973). Dopamine and noradrenaline receptor stimulation: reversal of reserpine induced suppression of motor activity. *Psychopharmacologia, Berl.*, **29**, 289–298.

CALNE, D.B., LEIGH, P.N., TEYCHENNE, P.F., BAMJI, A.N. & GREENACRE, J.K. (1974). Treatment of parkinsonism with bromocriptin. *The Lancet*, Dec. 7, 1355–1356.

CALNE, D.B., TEYCHENNE, P.F., CLAVERIA, L.E., EASTMAN, R., GREENACRE, J K & PETRIE, A. (1974). Bromocriptin in parkinsonism. *Br. Med. J.*, **4**, 442–444.

CARLSSON, A., HILLARP, N.-A. & WALDECK, B. (1963). Analysis of the Mg^{2+}-ATP dependent storage mechanism in the amine granules of the adrenal medulla. *Acta physiol. scand.*, **59**, suppl. 215, 1–38.

CARLSSON, A., LINDQVIST, M & MAGNUSSON, T (1957). 3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists. *Nature, Lond.*, **180**, 1200.

CORRODI, H , FUXE, K., HÖKFELT, T., LIDBRINK, P. & UNGERSTEDT, U. (1973). Effects of ergot drugs on central neurons: evidence for a stimulation of central dopamine neurons. *J. Pharm. Pharmac.*, **25**, 409–410.

COSTA, E., GROPPETTI, A. & NAIMZADA, M.K (1972). Effects of amphetamine on the turnover rate of brain catecholamines and motor activity. *Br. J. Pharmac.*, **44**, 742–751.

COSTALL, B., NAYLOR, R.J. & OLLEY, J.E. (1972). Stereotypic and anti-cataleptic activities of amphetamine after intracerebral injections. *Eur. J. Pharmac.*, **18**, 83–94.

DEL POZO, E., BRUN DEL RE, R , VORGA, L. & FIESEN, H. (1972). The inhibition of prolactin secretion in man by CB-154 (2-Br-α-ergokryptine). *J. clin. Endocr. Metab.*, **35**, 768–771.

DINGWELL, J V., OWENS, M.L., NORWICH, R. & SULSER, F. (1967). On the role of norepinephrine biosynthesis in the central action of amphetamine. *Life Sciences*, **6**, 1155–1162.

DOMINIC, J.A. & MOORE, K.E. (1969). Supersensitivity to the central stimulant actions of adrenergic drugs following discontinuation of a chronic diet of α-methyl-*p*-tyrosine. *Psychopharmacologica, Berl.*, **15**, 96–101.

ERNST, A.M. (1967). Mode of action of apomorphine and dexamphetamine in gnawing compulsion in rats. *Psychopharmacologia, Berl.*, **10**, 316–323.

ERNST, A.M. & SMELIK, P. (1966). Site of action of dopamine and apomorphine on compulsive gnawing behaviour in rats. *Experientia*, **22**, 837–838.

FLÜCKIGER, E. (1972). Drugs and the control of prolactin secretion. In: *Prolactin and carcinogenesis*, ed. Boyns, A.R. and Griffiths, K. Cardiff: Alpha Omega Alpha Publishing.

FOG, R.L., RANDRUP, A. & PAKKENBERG, H (1967). Aminergic mechanisms in corpus striatum and amphetamine induced stereotyped behaviour. *Psychopharmacologia, Berl.*, **11**, 179–183.

FUXE, K. & UNGERSTEDT, U. (1968). Histochemical studies on the effect of (+)-amphetamine, drugs of the imipramine group and tryptamine on central catecholamine and 5-hydroxytryptamine neurons after intraventricular injection of catecholamines and 5-hydroxytryptamine. *Eur. J. Pharmac.*, **4**, 135–144.

HANSON, L.C.F. (1966). Evidence that the central action of amphetamine is mediated via catecholamines. *Psychopharmacologia, Berl.*, **9**, 78–80.

HOKFELT, T & FUXE, K. (1972). On the morphology and the neuroendocrine role of the hypothalamic catecholamine neurons. In: *Brain-endocrine interactions*, ed. Knigge, K.M., Scott, D.E. and Weindl, A. Basle: Karger.

JOHNSON, A.M., VIGOURET, J-M. & LOEW, D M. (1973). Central dopaminergic actions of ergotoxine alkaloids and some derivatives. *Experientia*, **29**, 763.

JOHNSON, A.M., VIGOURET, J.M. & LOEW, D M (1974) CB 154 (2-bromo-ergokryptine, Bromocriptin), a potential anti-parkinson agent. *Naunyn-Schmiedebergs Arch. Pharmak.*, **282**, Suppl. R40.

MAJ, T, GRABOWSKA, M. & GAJDA, L. (1972). Effects of apomorphine on motility in rats. *Eur. J. Pharmac.*, **17**, 208–214.

MILLER, L C. & TAINTER, M.L (1944). Estimation of the ED$_{50}$ and its error by means of logarithmic-probit graph paper. *Proc. Soc. exp. Biol. Med.*, **57**, 261–264.

MORPURGO, C & THEOBALD, W (1964). Influence of anti-Parkinson drugs and amphetamine on some pharmacological effects of phenothiazine derivatives used as neuroleptics. *Psychopharmacologia, Berl.*, **6**, 178–191.

MORPURGO, C. & THEOBALD, W. (1966). Behavioural reactions to amphetamine in reserpinised rats. *Int. J. Neuropharmac.*, **5**, 375–377.

PASTEELS, J.J., DANGUY, A., FREROTTE, M. & ECTORS, F. (1971). Inhibition de la secretion de prolactine par l'ergocornine et la 2-brome-α-ergocryptine. Action directe sur l'hypophyse en culture. *Annls. Endocr.*, **32**, 188–211.

QUINTON, R. & HALLIWELL, G. (1963). Effects of α-methyldopa and dopa on the amphetamine excitatory response in reserpinised rats. *Nature, Lond.*, **200**, 178–179.

RANDRUP, A. & MUNKVAD, I. (1968). Behavioural stereotypies induced by pharmacological agents. *Pharmakopsychiatrie, Stuttgart*, **1**, 18–26.

SMITH, C.B. (1963). Enhancement by reserpine and α-methyl-DOPA of the effects of d-amphetamine upon locomotor activity of mice. *J. Pharmac. exp. Ther.*, **142**, 343–350.

SPECTOR, S., SJOERDSMA, A. & UNDENFRIEND, S. (1965). Blockade of endogenous norepinephrine synthesis by α-methyl-*p*-tyrosine, an inhibitor of tyrosine hydroxylase. *J Pharmac. exp. Ther.*, **147**, 86–95.

STOLK, J.M. & RECH, R.H. (1968). Enhanced stimulant effects of d-amphetamine in rats treated chronically with reserpine. *J. Pharmac. exp. Ther.*, **163**, 75–83.

SVENSSON, T.H. & WALDECK, B. (1970). On the role of brain catecholamines in motor activity: experiments with inhibitors of synthesis and of monoamine oxidase. *Psychopharmacologia, Berl.*, **18**, 357–365.

UNGERSTEDT, U. (1971). Striatal dopamine release after amphetamine or nerve degeneration revealed by rotational behaviour. *Acta. physiol. scand., suppl.*, **387**, 49–93.

VAN ROSSUM, J.M. (1970). Mode of action of psychomotor stimulant drugs. *Int. J. Neurobiol.*, **12**, 307–383.

VAN ROSSUM, J M & HURKMANS, J.A. (1964). Mechanism of action of psychomotor stimulant drugs. Significance of dopamine in locomotor stimulant action. *Int. J. Neuropharmac.*, **3**, 227–239.

VAN ROSSUM, J M., JANSSEN, P.A.J., BOISSIER, J.R., JULOU, L, LOEW, D.M., MØLLER-NIELSEN, I., MUNKVAD, I., RANDRUP, A, STILLE, G. & TEDESCHI, D.H (1970). *Pharmacology, The Neuroleptics. Mod. Probl. Pharmacopsychiat.*, ed. Bobon, D.P. and Janssen, P.A.J., Vol. 5, pp. 23–70. Basle: Karger.

WEISSMAN, A.K., KOE, B. & TENEN, S.S. (1966). Anti-amphetamine effects following inhibition of tyrosine hydroxylase. *J. Pharmac. exp. Ther.*, **151**, 339–352.

ZETLER, G. & MOOG, E. (1958). Die Bulbocapnin-Katatonie, ihre Synergisten und Antagonisten. *Naunyn-Schmeidebergs. Pharmak.*, **232**, 442–458.

(Received May 30th, 1975)

Br. J. Pharmac. (1976), **56**, 69–75

PROLONGED EFFECTS OF *p*-CHLOROPHENYLALANINE ON THE BLOOD PRESSURE OF CONSCIOUS NORMOTENSIVE AND DOCA/SALINE HYPERTENSIVE RATS

R.E. BUCKINGHAM, T.C. HAMILTON & R.A. MOORE[1]
Pharmacology Department, Roche Products Limited, Welwyn Garden City, Hertfordshire

1 In deoxycorticosterone acetate (DOCA) saline hypertensive rats a single dose of *p*-chlorophenylalanine methylester (PCPAME) (400 mg/kg i.p.) produced a significant fall in blood pressure (20–43 mmHg) which lasted for at least 8 days and was accompanied by a parallel depletion of brain stem 5-hydroxytryptamine (5-HT) but not of noradrenaline (NA).

2 In normotensive rats single doses of PCPAME (200 and 400 mg/kg i.p.) produced a significant hypotension (15–20 mmHg) after a latent period of 5 days. An initial pressor response (12 mmHg) was observed at the higher dose level only on day 3.

3 The hypotensive response to PCPAME (200 mg/kg i.p.) in normotensive rats was not modified by pretreatment with 5,6-dihydroxytryptamine (5,6-DHT; 50 μg i.c.v.) or 6-hydroxydopamine (6-OHDA; 3 x 250 μg intracerebroventricularly).

4 It is concluded that the hypotensive response to PCPAME in normotensive rats is independent of brain stem depletion of 5-HT and is probably not mediated by the formation of a false transmitter substance acting via central noradrenergic inhibitory pathways. The mechanism involved in the antihypertensive response to PCPAME in DOCA/saline hypertensive rats has yet to be defined.

Introduction

Serotoninergic neurones have been identified in various vasomotor regions of the central nervous system (Dahlström & Fuxe, 1965; Fuxe, Hökfelt & Ungerstedt, 1968) but their rôle in cardiovascular regulation is poorly understood. Central serotoninergic mechanisms in circulatory control have been investigated in only a few studies with conflicting results. Intracisternal administration of 5,6-dihydroxytryptamine (5,6-DHT), which produces a selective destruction of central 5-hydroxytryptamine (5-HT) secreting nerve terminals (Baumgarten, Lachenmayer & Schlossberger, 1972) causes a fall in blood pressure in normal and in neurogenically hypertensive rabbits, and prevents the development of neurogenic, but not of renal hypertension in this species (Wing & Chalmers, 1974a). The development and maintenance of deoxycorticosterone acetate (DOCA)/saline hypertension in the rat were unaffected by intracisternal injections of 5,6-DHT (Myers, Reid & Lewis, 1974) but the rate of development of hypertension in genetically hypertensive rats was retarded by the intracerebroventricular (i.c.v.) injection of 5,6-DHT to 6 week old animals (Buckingham, Hamilton & Robson, unpublished results). Parachlorophenylalanine (PCPA) depletes 5-HT stores by inhibition of tryptophan hydroxylase (Koe & Weissman, 1966; Jéquier, Lovenberg & Sjoerdsma, 1967) and although it does not prevent the onset of neurogenic hypertension in the rabbit, it lowers blood pressure in the normotensive rabbit (Wing & Chalmers, 1974b) and in the genetically hypertensive rat (Jarrot, McQueen and Louis, 1975). In contrast, PCPA raises blood pressure in the normotensive rat (Ito and Schanberg, 1972) and is without effect in the normotensive dog (Dunkley, Sanghvi, Friedman & Gershon, 1972).

In conscious dogs, moderate to high doses of 5-hydroxytryptophan (5-HTP) produce an elevation of blood pressure (Bogdanski, Weissbach & Udenfriend, 1958; Sanghvi & Gershon, 1970) whereas a hypotensive response is produced in conscious rats (Henning & Rubenson, 1971) and anaesthetized, intact monoamine oxidase inhibited cats (Florez & Armijo, 1974) and anaesthetized monoamine oxidase inhibited dogs (Antonaccio & Robson, 1973). In the present studies, the effects of DL-*p*-chlorophenylalanine methyl ester hydrochloride (PCPAME) on resting blood pressure have been investigated in conscious normotensive and DOCA/saline hypertensive rats.

---

[1] Present address: Servier Laboratories, Greenford, Middlesex.

Methods

Induction of experimental hypertension

Experimental hypertension was induced in male Sprague-Dawley rats, 120–150 g, by unilateral nephrectomy under ether anaesthesia and subcutaneous implantation of 2 compressed tablets each containing 25 mg deoxycorticosterone acetate (DOCA). The drinking water was replaced by 0.9% w/v saline. Studies were begun 5–6 weeks later. Animals with a systolic blood pressure of less than 180 mmHg (measured indirectly: see below) were not included in the study.

Indirect measurement of systolic blood pressure

Rats were placed in an incubator (32–34°C) for 30 min to 1 hour. Systolic blood pressure (1 mmHg 133 Pa) was then measured indirectly using a tail cuff strain gauge detector coupled to a W & W 8002 recorder.

Intra-cerebroventricular (i.c.v.) administration of drugs

Some normotensive animals received i.c.v. injections via a cannula implanted in the lateral cerebral ventricle according to the method of Hayden, Johnson & Maickel (1966). Cannulae were implanted approximately 1 week before studies commenced.

Drug administrations

a. The effects of single doses of PCPAME (200 and 400 mg/kg), administered intraperitoneally (i.p.), were studied in normotensive and DOCA/saline hypertensive rats. Systolic blood pressure was monitored 6, 4, 2 days and immediately before dosing (day 0) and 1,3, 5, 8, 11 and, in some experiments, 16 days after dosing.

b. In a separate experiment, 5-HT and noradrenaline (NA) levels in medulla-pons were determined 1, 3, 5, 8 and 11 days after PCPAME (400 mg/kg i.p.), administered to DOCA/saline hypertensive rats.

c. Normotensive rats were pretreated with 5,6-DHT (50 µg i.c.v.), or vehicle, 4 days before PCPAME (200 mg/kg i.p.). Systolic blood pressure was monitored as described in *a.*

d. Normotensive rats were pretreated with 6-hydroxydopamine (6-OHDA; 250 µg i.c.v.), or vehicle, 6, 4 and 2 days before PCPAME (200 mg/kg i.p.). Systolic blood pressure was monitored as described in *a.*

Biochemical determination of 5-HT and noradrenaline (NA) in medulla-pons

Rats were killed by decapitation. Brains were removed and placed on a cooled glass plate. The medulla-oblongata-pons region was dissected out according to the method of Miller, Cox, Snodgrass & Maickel (1970). This procedure took less than 2 min to perform. The brain samples were frozen in polythene bags at −20°C until analyses were performed, usually within 1 day. The brain samples were pooled in groups of 4 or 5 and homogenized in 7 ml of cold 0.4 N perchloric acid containing EDTA (0.1%), and ascorbic acid (0.2 mg/ml). The extraction procedure for NA and 5-HT was essentially that of Uretsky & Iversen (1970). After the potassium hydroxide neutralization step, the supernatant and precipitate washings were combined, shaken and divided into 2 equal parts, one for NA determination and the other for 5-HT. Column chromatography was employed to purify and concentrate the extracts. Noradrenaline was adsorbed on Amberlite CG 120 Na^+ form cation exchange resin, and eluted with 10 ml of 1 N HCl (Uretsky & Iversen, 1970) whilst 5-HT was adsorbed on Amberlite CG 50 Na^+ form resin, and eluted with 3 ml of 1 N HCl (Anden & Magnusson, 1967). Aliquots of the eluates were estimated for NA by the method of Laverty and Taylor (1968). The fluorescence technique of Anden & Magnusson (1967) was used to measure 5-HT. Results were corrected for loss of amines during the isolation procedure by inclusion of internal standards. Average recoveries for NA and 5-HT were 80% and 65% respectively.

Drugs

For intraperitoneal injection: DL-*p*-chlorophenylalanine methyl ester hydrochloride (PCPAME) (Koch-Light) dissolved in 0.9% saline. Doses are expressed as the salt.

For i.c.v. injection: 6-hydroxydopamine hydrobromide (6-OHDA) dissolved in nitrogen bubbled N/100 hydrochloric acid; 5,6-dihydroxytryptamine creatinine sulphate (5,6-DHT) dissolved in 0.1 mg/ml ascorbic acid. Both drugs were kindly supplied by Dr Langemann (F. Hoffmann La Roche Limited, Basel). Doses of these drugs are expressed as the base.

Statistical analysis

Changes in systolic blood pressure for each animal at each time interval were related to values recorded on day 0. Group mean changes were calculated together

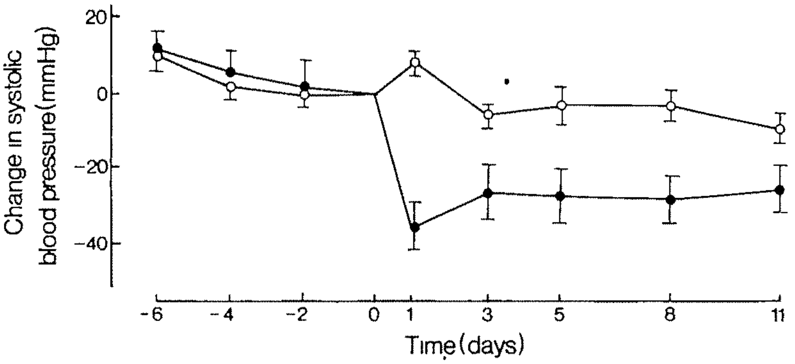


Figure 1 Time course (days) of change in systolic blood pressure (mmHg) produced by (●) PCPAME, 400 mg/kg i.p. and by (○) vehicle, injected at day 0, in DOCA/saline hypertensive rats. At day 0 the resting systolic blood pressure of the treated ($n=22$) and control ($n=18$) groups were, respectively, 216 ± 5 and 217 ± 4 mmHg

n is the number of animals per group, vertical bars extend to s.e. mean.

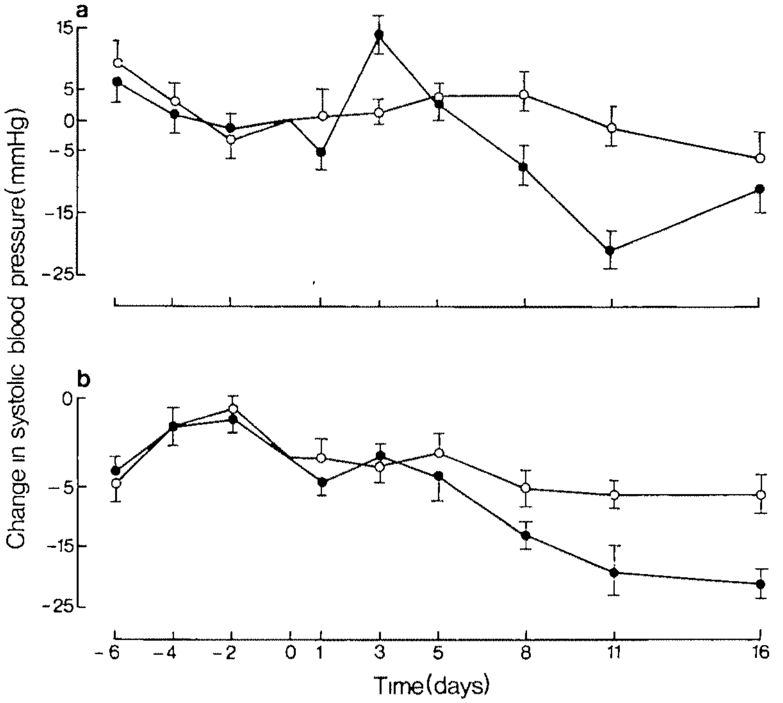


Figure 2 Time-course (days) of change in systolic blood pressure (mmHg) produced (a) by PCPAME, 400 mg/kg i.p (●), and vehicle (○) and (b) by PCPAME, 200 mg/kg i.p (●), and vehicle (○) injected at day 0 in normotensive rats. At day 0 the resting systolic blood pressure of treated and control groups were (a) 142 ± 3 ($n=16$) and 144 ± 3 ($n=12$) mmHg respectively and (b) 144 ± 1 ($n=15$) and 145 ± 2 ($n=15$) mmHg respectively.

n is the number of animals per group; vertical bars extend to s.e mean

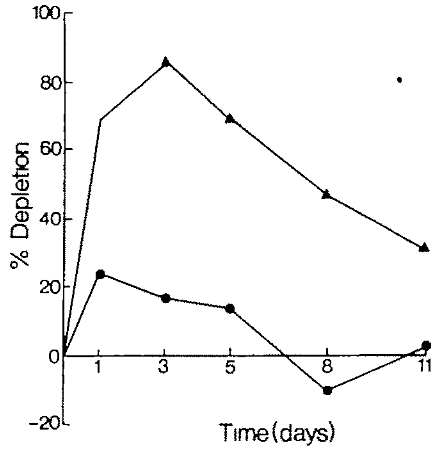


Figure 3 Time-course (days) of percentage depletion of NA (●) and 5-HT (▲) from the medulla-pons region of DOCA/saline hypertensive rats treated at day 0 with PCPAME (400 mg/kg i.p.). Each point represents the mean of determinations carried out in groups of 4 or 5 rats at each time interval

with standard error of the mean. Differences between groups were assessed by Student's 't' test.

Results

Effects of single doses of PCPAME on the blood pressure of DOCA/saline hypertensive and normotensive rats

In DOCA/saline hypertensive rats PCPAME (400 mg/kg i.p.) produced a significant fall in systolic blood pressure (20–43 mmHg) which was maintained for at least 8 days after drug administration (Figure 1). In other experiments PCPAME (200 mg/kg i.p.) produced no significant changes in systolic blood pressure.

In normotensive rats single doses of PCPAME (200 and 400 mg/kg i.p.) also produced significant falls in systolic blood pressure though only after a latent period of 5 days (Figures 2a and 2b). During this initial period, PCPAME (400 mg/kg, but not 200 mg/kg i.p.) produced a significant rise in systolic blood pressure on day 3 (12 mmHg); the hypotensive response following the higher dose reached a maximum on day 11 (20 mmHg) and the blood pressure was not significantly different from that of the control group by day 16. The hypotensive response evoked by PCPAME (200 mg/kg i.p.) was greatest on day 16 (15 mmHg) when the study was terminated.

Effect of a single dose of PCPAME (400 mg/kg i.p.) on 5-HT and NA levels in medulla-pons region of the brain of DOCA/saline hypertensive rats

PCPAME, 400 mg/kg i.p., in DOCA/saline hypertensive rats produced a rapid depletion of 5-HT in the medulla-pons region (Figure 3). Maximal depletion (85%) was achieved by day 3 and thereafter 5-HT levels gradually returned towards control levels, though 5-HT levels were still 30% depleted by day 11. PCPAME also slightly decreased NA levels (24% on day 1) but by day 8 NA levels in PCPAME-treated rats were slightly higher (10%) than those observed in control animals.

Effect of pretreatment with 5,6-DHT (50 µg i.c.v.) on the delayed hypotensive response to PCPAME (200 mg/kg i.p.) in normotensive rats

Pretreatment of a group of normotensive rats with 5,6-DHT (50 µg i.c.v.) produced no significant change in systolic blood pressure when compared to a group pretreated with vehicle only (Figure 4). Four days

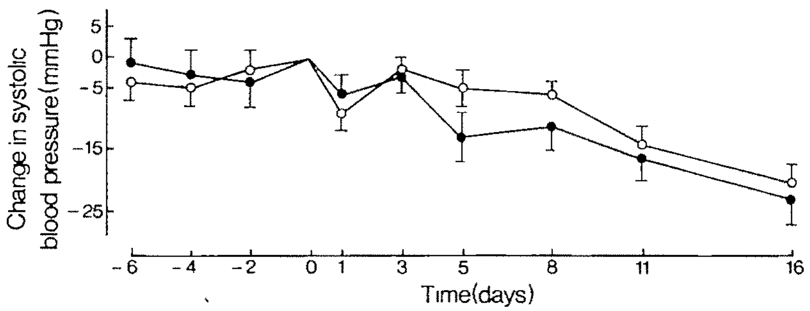


Figure 4 Time-course (days) of change in systolic blood pressure (mmHg) produced by PCPAME (200 mg/kg i.p.), injected at day 0, in normotensive rats pretreated with i.c.v 5,6-DHT (50 µg ●) or vehicle (O) at day-4. At day 0, the resting systolic blood pressure of 5,6-DHT treated rats ($n=22$) and control rats ($n=23$) were, respectively, 139 ± 3 and 138 ± 2 mmHg.
n is the number of animals per group, vertical bars extend to s.e. mean.

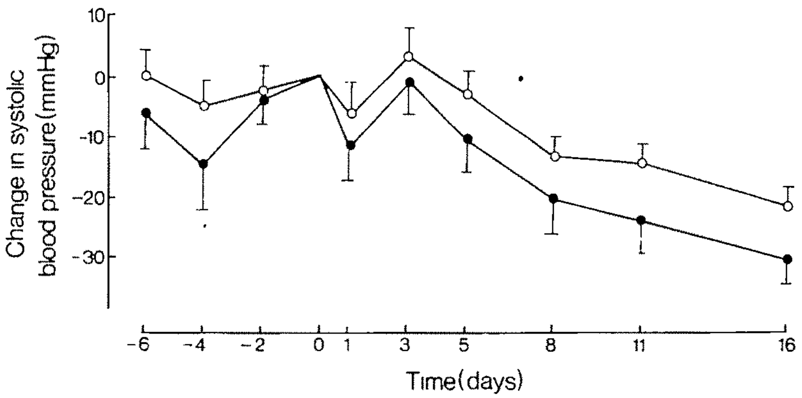


Figure 5 Time-course (days) of change in systolic blood pressure (mmHg) produced by PCPAME (200 mg/kg i.p.), injected at day 0, in normotensive rats pretreated with i.c.v. 6-OHDA (250 µg; ●) or vehicle (○) on days −6, −4 and −2 At day 0, the resting systolic blood pressure of 6-OHDA-treated rats (*n* = 13) and control rats (*n* = 15) were, respectively, 151 ± 5 and 146 ± 3 mmHg.
n is the number of animals per group; vertical bars extend to s e mean.

after pretreatment all rats received PCPAME (200 mg/kg i.p.). The pattern of the blood pressure response to PCPAME was similar in both groups though in the 5,6-DHT-pretreated group the onset of the hypotensive response preceded that in the control group. The magnitude of the hypotensive response recorded on days 11 (14–17 mmHg) and 16 (20–23 mmHg) were very similar.

Effect of pretreatment with 6-OHDA (3 × 250 µg) on the delayed hypotensive response to PCPAME (200 mg/kg i.p.) in normotensive rats

Pretreatment of a group of normotensive rats with 6-OHDA (3 × 250 µg i.c.v.) produced a slight fall in systolic blood pressure following the first dose but subsequent recovery to resting levels on day 0. Pretreatment with vehicle (i.c.v.) produced little effect on systolic blood pressure. Following pretreatment with 6-OHDA, rats developed characteristic symptoms of aggression. The pattern of the blood pressure response to PCPAME was similar in both groups (Figure 5), and at no time interval were the blood pressure changes of the 2 groups significantly different.

Discussion

These investigations have shown that single doses of PCPAME produced a prolonged hypotensive response in both conscious normotensive and DOCA/saline hypertensive rats; in addition a small pressor response was recorded after PCPAME (400 mg/kg), in normotensive rats. Reductions in arterial blood pressure have also been observed in the normotensive rabbit (Wing & Chalmers, 1974b) and genetically hypertensive rat (Jarrot *et al.*, 1975) when PCPA was administered in repeated dose studies. Ito & Schanberg (1972) have previously demonstrated that the elevation of blood pressure produced by PCPA in normotensive rats was prevented, or could be reversed, by treatment with 5-HTP; in addition a hypotensive phase was observed 5 days after the administration of PCPA (200 mg/kg), but the effect was short-lived. Ito & Schanberg (1972) also found that the hypertensive response to PCPA in normotensive rats was accompanied by marked parallel depletion of brain 5-HT levels. In the present study the antihypertensive response produced by PCPAME (400 mg/kg) in DOCA/saline hypertensive rats was accompanied by a parallel depletion of 5-HT, but not of NA, from the brain stem and followed a similar time course to that described by Miller *et al.* (1970) during studies with PCPA (400 mg/kg) in normotensive rats. It appears therefore that in normotensive rats the significant rise in blood pressure observed on day 3 in our experiments, coincided with the maximal degree of 5-HT depletion in the medulla-pons and is thereby in agreement with the observations of Ito & Schanberg (1972).

In separate experiments 5-HTP (150–200 mg/kg) subcutaneously, was administered to DOCA/saline hypertensive rats, both in the presence and absence of the peripheral decarboxylase inhibitor, Ro 04-4602 (50 mg/kg i.p.) in an attempt to restore 5-HT levels depleted by PCPAME. The experiments, however, were inconclusive since 5-HTP *per se* produced an antihypertensive response.

We are unable, at present, to demonstrate if the antihypertensive response in DOCA/saline hypertensive rats produced by PCPAME is a function of brain 5-HT depletion.

In contrast to the studies of Ito & Schanberg (1972) the elevation of systolic blood pressure produced in normotensive rats by PCPAME in our experiments was transient and small relative to the ensuing hypotensive phase; a smaller dose of PCPAME (200 mg/kg i.p.) did not produce an elevation of systolic blood pressure. The dominant hypotensive phase produced by both doses of PCPAME would appear, from the biochemical study, to be independent of depletion of 5-HT in the brain stem. This conclusion is supported by the finding that in rats pretreated with 5,6-DHT (50 µg) the hypotensive response to PCPAME (200 mg/kg i.p.) was not significantly modified. Baumgarten, Björkland, Lachenmayer, Nobin & Stenevi (1971) showed that 5,6-DHT (25–75 µg) injected into the lateral cerebral ventricle of the rat produced a marked and persistent depletion of brain 5-HT; whilst depletion of 5-HT was lowest in medulla-pons, other areas and especially spinal cord were markedly depleted. Baumgarten *et al.* (1972) later showed that 5,6-DHT (75 µg) not only depletes 5-HT stores but also causes degeneration of serotoninergic nerve endings. If the hypotensive response to PCPAME in normotensive animals was a consequence of central 5-HT depletion, then treatment with 5,6-DHT itself might have been expected to produce a hypotensive response.

In our experiments 5,6-DHT did not produce any detectable change in systolic blood pressure. Similar observations have been made in DOCA/saline hypertensive rats when 5,6-DHT was administered intracisternally (Myers *et al.*, 1974), and when administered intracerebroventricularly to adult genetically hypertensive rats (Buckingham, Hamilton & Robson, unpublished results). It is possible that bundles of central serotoninergic neurones which may mediate the response to PCPA may be insensitive to this regimen of 5,6-DHT treatment.

Stark & Fuller (1972) suggested that some of the effects of PCPA on blood pressure may be mediated by a metabolite, *m*-chlorotyrosine, which may in turn be converted to a false transmitter, *m*-chloro-octopamine. King & Caldwell (1974) showed that, in the cat, *m*-chlorotyrosine induced, after a brief latent period, an elevation of blood pressure. This interesting observation raised the possibility that the prolonged hypotensive response to PCPAME in normotensive rats may be due to the release of an, as yet unidentified, false transmitter substance which acts centrally. The persistence of the response to PCPAME after destruction of central noradrenergic inhibitory pathways by 6-OHDA, suggests that a false transmitter acting through central noradrenergic inhibitory pathways is unlikely to mediate the hypotensive response to PCPAME in normotensive rats. The same pretreatment regimen has been shown in other studies to produce a widespread destruction of the nerve terminals of central noradrenergic neurones (Uretsky & Iversen, 1970) and to inhibit the antihypertensive response to α-methyldopa in genetically hypertensive rats (Finch & Hauesler, 1973). There remains the possibility that bundles of noradrenergic neurones involved in such a mediated response could be insensitive to this pretreatment regimen of 6-OHDA. At present we are unable to define the mechanism by which PCPAME produces prolonged hypotension in normotensive rats. The evidence so far accumulated suggests that this response is independent of brain stem 5-HT depletion.

The authors are indebted to Mr K. Dickinson for performing the biochemical determinations and to Mr K. Shaw for his technical assistance.

References

ANDEN, N-E. & MAGNUSSON, T (1967). An improved method for the fluorimetric determination of 5-hydroxytryptamine in tissues. *Acta physiol. scand.*, **69**, 87–94.

ANTONACCIO, M.J. & ROBSON, R.D. (1973). L-dopa hypotension: evidence for mediation through central 5-HT release. Cardiovascular effects of 5-hydroxytryptophan in MAO-inhibited anaesthetized dogs. *Pharmacologist*, **15**, 179.

BAUMGARTEN, H.G., BJÖRKLAND, A., LACHENMAYER, L, NOBIN, A. & STENEVI, U. (1971). Long-lasting selective depletion of brain serotonin by 5,6-dihydroxytryptamine. *Acta physiol. scand.*, **84**, suppl. 373, 1–15.

BAUMGARTEN, H.G., LACHENMAYER, L. & SCHLOSSBERGER, H.G. (1972). Evidence for a degeneration of indoleamine containing nerve terminals in rat brain, induced by 5,6-dihydroxytryptamine. *Z. Zellforsch.* **125**, 553–569.

BOGDANSKI, D.F., WEISSBACH, H. & UDENFRIEND, S (1958). Pharmacological studies with the serotonin precursor, 5-hydroxytryptophan. *J. Pharmac. exp. Ther.*, **122**, 182–194.

DAHLSTRÖM, A & FUXE, K. (1965). Evidence for the existence of monoamine neurons in the central nervous system: II. Experimentally induced changes in the intraneuronal amine levels of bulbospinal neuron systems. *Acta physiol. scand.*, **64**, suppl. 247, 1–36.

DUNKLEY, B., SANGHVI, I., FRIEDMAN, E. & GERSHON,

S. (1972). Comparison of behavioural and cardiovascular effects of L-dopa and 5-HTP in conscious dogs. *Psychopharmacologia (Berl.)*, **26**, 161–172.

FINCH, L. & HAUESLER, G. (1973). Further evidence for a central hypotensive action of α-methyldopa in both the rat and cat. *Br. J. Pharmac.*, **47**, 217–228.

FLOREZ, J. & ARMIJO, J.A. (1974). Effect of central inhibition of the 1-amino acid decarboxylase on the hypotensive action of 5-HT precursors in cats. *Eur. J. Pharmac.*, **26**, 108–110.

FUXE, K., HÖKFELT, T. & UNGERSTEDT, U. (1968). Localisation of indolealkylamines in CNS. *Advances in Pharmacology*, **6A**, 235–251.

HAYDEN, J.F., JOHNSON, L.R. & MAICKEL, R.P. (1966). Construction and implantation of a permanent cannula for making injections into the lateral ventricle of the rat brain. *Life Sci.*, **5**, 1509–1515.

HENNING, M. & RUBENSON, A. (1971). Effects of 5-hydroxytryptophan on arterial blood pressure, body temperature and tissue monoamines in the rat. *Acta pharmac. toxicol.*, **29**, 145–154.

ITO, A. & SCHANBERG, S.M. (1972). Central nervous system mechanisms responsible for blood pressure elevation induced by p-chlorophenylalanine. *J. Pharmac. exp. Ther.*, **181**, 65–74.

JARROT, B., McQUEEN, A. & LOUIS, W.J. (1975). Levels of serotonin in brain and vascular tissue and the importance of serotonin in blood pressure control. *Clin. exp. Pharmacol. Physiol.* Suppl. 2, 201–205.

JÉQUIER, E., LOVENBERG, W. & SJOERDSMA, A. (1967). Tryptophan hydroxylase inhibition: the mechanism by which p-chlorophenylalanine depletes rat brain serotonin. *Molec. Pharmac.*, **3**, 274–278.

KING, C.D. & CALDWELL, R.W. (1974). Pressor response induced by meta-chlorotyrosine in cats. *Pharmacologist*, **16**, No. 2, 280.

KOE, B.K. & WEISSMAN, A. (1966) p-Chlorophenylalanine: a specific depletor of brain serotonin. *J. Pharmac. exp. Ther.*, **154**, 499–516.

LAVERTY, R. & TAYLOR, K.M. (1968). The fluorometric assay of catecholamines and related compounds: improvements and extensions of the hydroxyindole technique. *Anal. Biochem.*, **22**, 269–279.

MILLER, F.P., COX, R.H., SNODGRASS, W.R. & MAICKEL, R.P. (1970). Comparative effects of p-chlorophenylalanine, p-chloroamphetamine and p-chloro-N-methylamphetamine on rat brain norepinephrine, serotonin and 5-hydroxyindole-3-acetic acid. *Biochem. Pharmac.*, **19**, 435–442.

MYERS, M.G., REID, J.L. & LEWIS, P.J. (1974). The effect of central serotonin depletion on DOCA/saline hypertension in the rat. *Cardiovascular Res.*, **8**, 806–810.

SANGHVI, I. & GERSHON, S. (1970). Similarities between behavioural and pharmacological actions of yohimbine and 5-hydroxytryptophan in the conscious dog. *Eur. J. Pharmac.*, **11**, 125–129.

STARK, P. & FULLER, R.W. (1972) Behavioural and biochemical effects of p-chlorophenylalanine, 3-chlorotyrosine and 3-chlorotyramine. A proposed mechanism for inhibition of self stimulation. *Neuropharmacology*, **11**, 261–272.

URETSKY, N.J. & IVERSEN, L.L. (1970). Effects of 6-hydroxydopamine on catecholamine containing neurones in the rat brain. *J. Neurochem.*, **17**, 269–278.

WING, L.M.H. & CHALMERS, J.P. (1974a). Participation of central serotonergic neurones in the control of the circulation of the unanaesthetised rabbit. *Circulation Res.*, **35**, 504–513.

WING, L.M.H. & CHALMERS, J.P. (1974b). Effects of p-chlorophenylalanine on blood pressure and heart rate in normal rabbits and rabbits with neurogenic hypertension. *Clin. exp. Pharmac. Physiol.*; **1**, 219–229.

(Received June 11, 1975)

Br. J. Pharmac. (1976), **56**, 77–85

EFFECTS OF DRUGS ACTING ON CEREBRAL 5-HYDROXYTRYPTAMINE MECHANISMS ON DOPAMINE-DEPENDENT TURNING BEHAVIOUR IN MICE

J.A. MILSON
Department of Pharmacology, University of Bristol Medical School, Bristol BS8 1TD
C.J. PYCOCK
Department of Neurology, Institute of Psychiatry, Denmark Hill, London SE5 8AF

1 The effects of drugs acting on cerebral 5-hydroxytryptaminergic mechanisms on drug-induced turning behaviour in mice with unilateral destruction of nigro-striatal dopaminergic nerve terminals have been studied.

2 Administration of L-tryptophan (400 mg/kg) or 5-hydroxytryptophan (200 mg/kg) increased brain 5-hydroxytryptamine and decreased the turning induced by both apomorphine (2 mg/kg) and amphetamine (5 mg/kg).

3 Parachlorophenylalanine (3 x 500 mg/kg) decreased brain 5-hydroxytryptamine and increased both apomorphine and amphetamine-induced circling behaviour.

4 Varying the protein content of dietary intake significantly altered brain 5-hydroxytryptamine and tryptophan levels, spontaneous locomotor activity and amphetamine-induced circling behaviour in these mice.

5 Systemic administration of methysergide (0.5–4 mg/kg), lysergic acid diethylamide (0.025–0.2 mg/kg), cyproheptadine (2.5–20 mg/kg) or clomipramine (0.6–20 mg/kg) produced no consistent effect on drug-induced turning behaviour.

6 The results suggest that circling behaviour due to striatal dopamine receptor stimulation is depressed by an elevation of brain 5-hydroxytryptamine and enhanced by a reduction in brain 5-hydroxytryptamine.

7 The possible physiological relationship between dopamine and 5-hydroxytryptamine neurones in the basal ganglia is discussed.

Introduction

Recent behavioural studies have stressed an interrelation between 5-hydroxytryptamine and the catecholamine transmitters in the brain (Hadžović & Ernst, 1969; Modigh, 1973a, 1974; Weiner, Goetz, Westheimer & Klawans, 1973; Jacobs, 1974; Jacobs, Eubanks & Wise, 1974). In rodents, an increase in dopamine receptor activity in one striatum as compared with the other causes the animal to turn in circles away from the side of higher activity (Andén, Dahlstrom, Fuxe & Larsson, 1966; Ungerstedt, 1971). In rats with unilateral nigro-striatal degeneration, but intact striatal dopamine receptors, directly acting dopamine agonists, such as apomorphine, induce circling away from the side of the lesion, because, it is suggested, the denervated receptors have become supersensitive (Ungerstedt, 1971). Indirectly acting dopamine agonists, such as amphetamine, cause release of dopamine from the nigro-striatal terminals of the intact side and cause the animal to circle towards the damaged side.

The basal ganglia is an area of the brain also rich in terminals of 5-hydroxytryptamine neurones (Fuxe, Hökfelt & Ungerstedt, 1968; Bak, Choi, Hassler, Usunoff & Wagner, 1975). The close anatomical relation between these two transmitter systems suggests a functional dependence; indeed 5-hydroxytryptamine has been shown to activate the release of dopamine in the rat striatum (Besson, Cheramy, Feltz & Glowinski, 1969) while dopamine receptor stimulation increases cerebral 5-hydroxytryptamine turnover (Grabowska, Antkiewicz, Maj & Michaluk, 1973).

In this study we have attempted to investigate the modulating role 5-hydroxytryptamine may exert on striatal dopamine function using the rotating mouse model of von Voigtlander & Moore (1973), a system that directly involves the dopaminergic neurones of the striatum. The effect of administering precursors of 5-hydroxytryptamine such as tryptophan and 5-hydroxytryptophan, of *p*-chlorophenylalanine, an inhibitor of tryptophan hydroxylase, and agents believed to block the 5-hydroxytryptamine receptor, has been studied on amphetamine and apomorphine-induced turning behaviour in mice with unilateral lesions of the nigro-striatal dopamine terminals.

Methods

Animals

Unilateral destruction of nigrostriatal dopamine terminals in male Swiss S strain mice was achieved by direct injection of 6-hydroxydopamine in a dose of 16 µg dissolved in 4 µl 0.9% w/v NaCl solution (saline) containing 0.2 mg/ml ascorbic acid, into the right striatum of a mouse under ether anaesthesia. The technique used was that described by von Voigtlander & Moore (1973), as modified by Pycock, Tarsy & Marsden (1976) for free hand injection. Mice were tested 2 days after operation with dexamphetamine sulphate (5 mg/kg) and those animals turning tightly towards the lesioned side were selected and tested at 10 days with apomorphine hydrochloride (2 mg/kg). Mice now turning away from the side of the lesion were selected for the following experiments.

Behavioural testing

Three groups of 20 mice were used to investigate the effect of systemic administration of 5-hydroxytryptophan (200 mg/kg), L-tryptophan (400 mg/kg) and *p*-chlorophenylalanine methyl ester (3 x 500 mg/kg) on amphetamine and apomorphine-induced circling behaviour. Ten mice from each group were randomly selected, marked and tested with apomorphine while the remainder were marked and tested with amphetamine. This enabled control turning rates of each group of mice to be determined. This procedure was repeated after 48 h when all control rates were found to be within 10% of those obtained previously. On the following day the 20-mouse groups were injected intraperitoneally with 5-hydroxytryptophan or L-tryptophan. The third group was injected three times at 24 hourly intervals with *p*-chlorophenylalanine. Amphetamine and apomorphine was administered to appropriate mice 30 min after 5-hydroxytryptophan or L-tryptophan and 16 h after the final injection of *p*-chlorophenylalanine. After injection of stimulant, all mice were placed in individual plastic boxes, measuring 12 x 12 x 8 cm. The number of complete revolutions made during a one-minute period was recorded 15 min after apomorphine (2 mg/kg) and 30 min after amphetamine (5 mg/kg). The number of counts in one minute was recorded for each group of mice and compared to the control values.

A further hour after counting, 10 mice from each of the three groups were randomly selected and killed, together with a group of 10 control animals, in order to determine brain 5-hydroxytryptamine levels.

For other pharmacological manipulations, groups of 20 mice were marked into subgroups of four for an incomplete Latin square design in order to randomize the distribution of drugs. Mice were tested on three separate days so that 12 observations were accumulated for each dose of drug. A saline-injected control group was run on each occasion. Methysergide, in a dose range 0.5–4 mg/kg and cyproheptadine, in a dose range 2.5–20 mg/kg, were administered 15 min before amphetamine and 30 min before apomorphine. Lysergic acid diethylamide (LSD) was administered in a dose range 25–200 µg/kg 5 min before amphetamine and 20 min before apomorphine.

In other experiments the effect of clomipramine in a dose range 0.6–20 mg/kg was investigated on submaximal turning in mice induced by 1.5 mg/kg amphetamine. As before an incomplete Latin square design was used, and animals were tested on three separate days. Clomipramine was administered intraperitoneally 30 min before amphetamine.

In a final series of experiments, the effect of a varying diet on the rate of turning induced by amphetamine was studied. Twenty mice were fed for two weeks on laboratory pellets (Dixons, Diet 41B). Throughout these experiments water was available *ad lib*. At the end of this period the mice were tested on three separate days with amphetamine (5 mg/kg) and divided into two equal groups so that the net turning rates of each group were the same. One group was fed high-protein (25%) pellets (Oxoid, breeding diet) while the other received a low protein diet composed of apple and potato. The groups were then tested weekly with amphetamine. Prior to amphetamine testing in the third week, spontaneous activity of the two groups was recorded in an Animex Activity recorder (LKB Farad Electronics). Following this third amphetamine testing the diet of the two groups was reversed, so that the low protein diet animals were now fed high protein pellets and *vice versa*. Animals were tested again at weekly intervals with amphetamine, and locomotor activity was recorded during the third week. At the end of this period mice from both diet groups were fed their normal diet of laboratory pellets (Diet 41B) and after one week the turning rate induced by 5 mg/kg amphetamine was observed. To complete the experiment, the 20 mice were randomly split into two groups again, fed the high and low protein diets for two weeks and then killed for brain biogenic amine and tryptophan estimation.

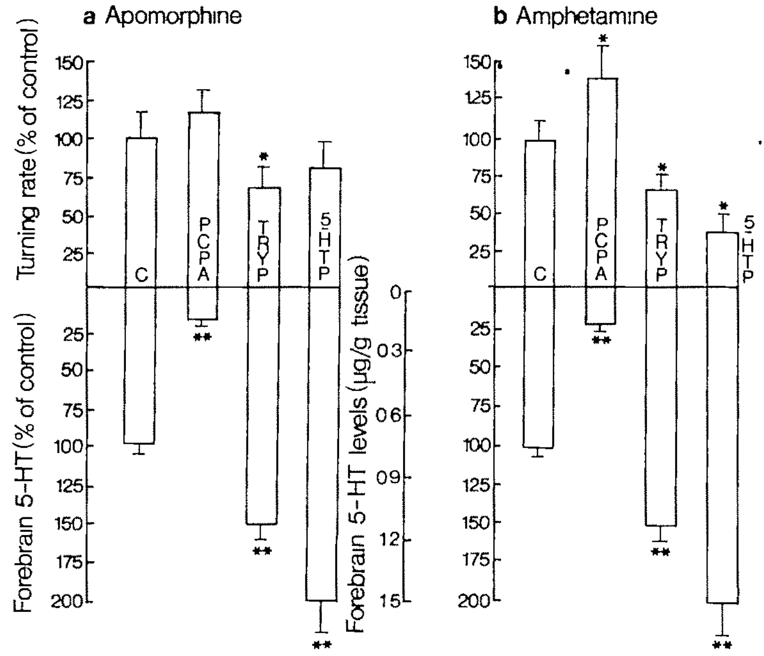


Figure 1 Effect of pretreatment of unilateral striatal lesioned mice with *p*-chlorophenylalanine (3 x 500 mg/kg) (PCPA), L-tryptophan (400 mg/kg) (Tryp), and 5-hydroxytryptophan (200 mg/kg) (5-HTP) on forebrain 5-hydroxytryptamine (5-HT) levels and on (a) apomorphine (2 mg/kg) and (b) amphetamine (5 mg/kg)-induced turning behaviour. C denotes control group of animals, and results are expressed as a percentage of control. Mean turning rates for control group were 7 6 ± 0.9, 30 min after amphetamine and 8.9 ± 0 7, 15 min after apomorphine Percentage means are shown for 10 observations of each drug Vertical lines show s.e mean. * Denotes statistical significance at the level $P < 0.05$ ** Denotes high statistical significance at the level $P < 0.001$.

Biochemical analyses

Forebrains from mice were weighed, homogenized in cold acidified butanol and extracted into HCl. 5-Hydroxytryptamine was assayed fluorimetrically after condensation with *o*-phthaldialdehyde (Maickel, Cox, Saillant & Miller, 1968); tryptophan was assayed by the method of Denckla & Dewey (1967), modified for brain tissue; noradrenaline was assayed fluorimetrically (Chang, 1964); and dopamine, after purification with alumina, was assayed by the method of Laverty & Sharman (1965).

Drugs

All agents were dissolved in saline, unless otherwise stated, and administered intraperitoneally in volumes no greater than 0.5 ml. 5-Hydroxytryptophan (Sigma Chemical Co) and L-tryptophan (Koch Light Chemicals Ltd) were dissolved in a little 2 N HCl and diluted with saline. The following drugs were used: *p*-chlorophenylalanine methyl ester (Sigma); methysergide bimaleate (Sandoz); cyproheptadine hydrochloride (Merck, Sharp & Dohme); lysergic acid diethylamide tartrate (Sandoz); clomipramine hydrochloride (Geigy); dexamphetamine sulphate (Smith, Kline & French); apomorphine hydrochloride (Evans Medical). Weights of drugs are expressed in terms of the salt.

Statistical analyses

Changes in turning rates were statistically compared using Student's *t* test.

Results

5-Hydroxytryptophan, L-tryptophan and p-chlorophenylalanine pretreatments

The effect of 5-hydroxytryptophan, L-tryptophan or *p*-chlorophenylalanine treatments on forebrain 5-hydroxytryptamine levels and turning rates induced by either apomorphine or amphetamine is shown in Figure 1. All agents caused significant changes in

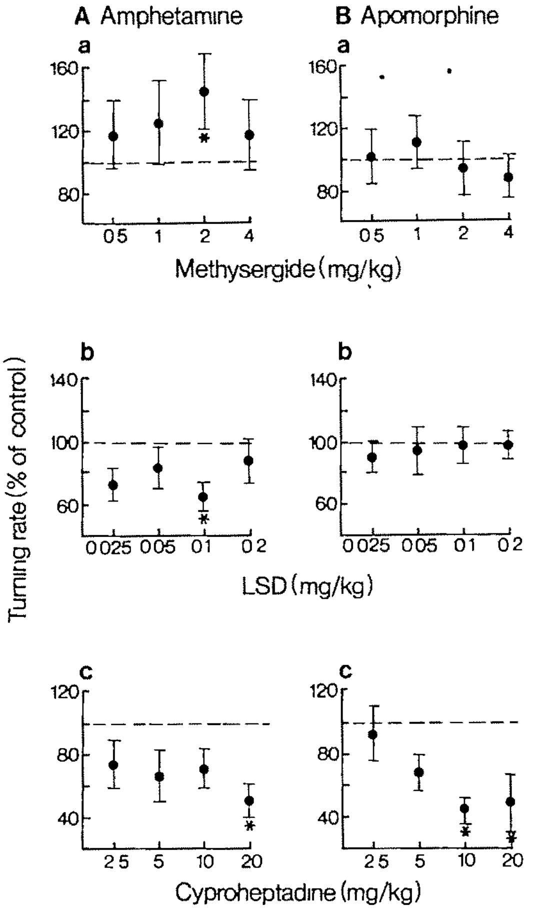


Figure 2 Effect of (a) methysergide (0.5–4 mg/kg), (b) Lysergic acid diethylamide (LSD, 0.025–0.2 mg/kg) and (c) cyproheptadine (2.5–20 mg/kg) on turning rates induced by amphetamine (5 mg/kg) (column A) and apomorphine (2 mg/kg) (column B). The mean number of turns per minute for each dose of drug is expressed as a percentage of the mean number of turns of the saline-treated control animals. The mean control turning rates were in the range 8.2–10.0 turns per min, 30 min after amphetamine and 8.3–10.7 turns per min, 15 min after apomorphine Means for 12 observations at each dose level are shown. Vertical lines show s.e mean. * Denotes statistical significance at the level $P<0.05$.

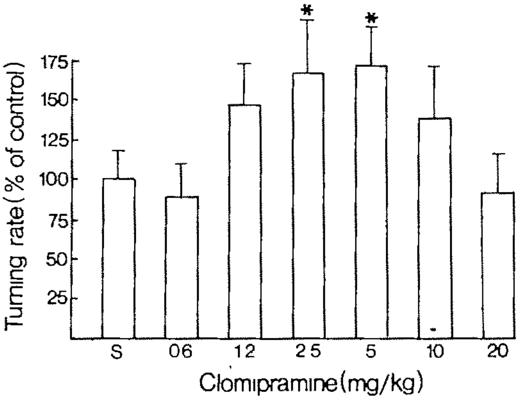


Figure 3 Effect of systematically administered clomipramine (0.6–20 mg/kg) on the rate of turning induced by amphetamine (1.5 mg/kg). The mean number of turns per min for each dose of clomipramine is expressed as a percentage of that of the saline-treated control group (S). The mean turning rate for the control group was 2.4±0.4 turns/min after amphetamine. Means for 10 observations at each dose level are shown. Vertical lines show s.e. mean * Denotes statistical significance at the level $P<0.05$.

brain 5-hydroxytryptamine levels, but alone did not induce turning behaviour or any marked body postures.

5-Hydroxytryptophan, in a single dose of 200 mg/kg, produced a 200% rise in 5-hydroxytryptamine content and a small but insignificant decrease in the rate of turning to apomorphine (to 80% of control values), but a greater and significant reduction in turning to amphetamine (to 40% of control values) ($P<0.01$). L-Tryptophan, in a single dose of 400 mg/kg, again elevated brain 5-hydroxytryptamine levels to 150% of control values and caused statistically significant depression of turning rates to about 70% of control counts for both apomorphine and amphetamine ($P<0.025$ and 0.05 respectively). Parachlorophenylalanine methyl ester, in three daily doses of 500 mg/kg, depleted cerebral 5-hydroxytryptamine levels to 19% of control values. Parachlorophenylalanine pretreatment caused a small but insignificant increase in the rate of turning to apomorphine (117% of control), and a larger and significant increase of amphetamine-induced turning (142% of control) ($P<0.05$).

Pharmacological agents

Methysergide (0.5–4 mg/kg) caused slight increases in amphetamine-induced turning rates, significant at the dose 2 mg/kg ($P<0.05$), but had no effect on the rate of apomorphine-induced turning (Figure 2). Methysergide alone evoked no circling behaviour and caused no postural asymmetries.

Cyproheptadine (2.5–20 mg/kg) produced depression of both apomorphine and amphetamine-induced turning. The highest dose (20 mg/kg) caused a significant ($P<0.05$) 50% reduction of turning behaviour to both drugs (Figure 2). Cyproheptadine

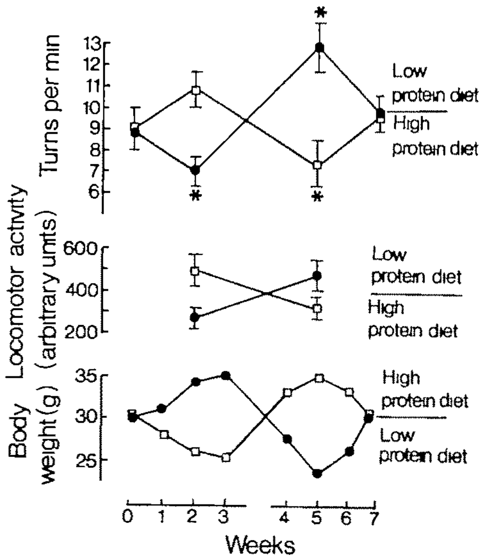


Figure 4 Effect of varying dietary protein intake on the rates of turning induced by amphetamine (5 mg/kg), on spontaneous locomotor activity and on body weight. Control turning rates were observed for two similar groups of mice. Group 1 mice (●) were then initially fed a high protein diet for 3 weeks, while Group 2 mice (□) received a low protein diet The diet was reversed for a further 3 weeks. At the end of this period both groups were fed normal laboratory diet Means for at least 10 observations at each time interval are shown. Vertical lines show s.e mean. * Denotes a statistically significant difference from the control result at the level $P < 0.05$.

alone evoked no circling behaviour or postural asymmetries: the higher doses caused sedation.

LSD (0.025–0.2 mg/kg) caused a slight depression of amphetamine-induced turning activity signfiicant at the dose 0.1 mg/kg ($P < 0.05$) while having no effect on apomorphine-induced turning (Figure 2). LSD alone showed little behavioural effect in the majority of animals. In the remainder mild degrees of stereotypy and hyperactivity occurred, but no consistent postural deviations or turning behaviour were observed.

Clomipramine (0.6–20 mg/kg) did not evoke a turning response and postural asymmetries were not seen; higher doses had a sedative effect. The drug's effect on a submaximal dose of amphetamine (1.5 mg/kg) is depicted in Figure 3. The middle doses tested (1.25, 2.5 and 5 mg/kg) potentiated amphetamine-induced turning (significant at the level $P < 0.05$ for the 2.5 and 5 mg/kg doses) while the lower and higher doses depressed it, but not to a statistically significant extent.

Diet experiments

The rates of turning induced by amphetamine in the two groups of mice fed high and low protein diets are shown in Figure 4. Dosage of amphetamine was carefully controlled to allow for the significant weight changes of the animals in the two groups. In general, mice fed a high protein diet (25% protein) showed a lower circling rate to amphetamine than did the mice fed a low protein diet (mainly carbohydrate). Both groups when fed the normal laboratory diet (15.9% protein) had a mean turning rate of 9.1 ± 0.9 turns/min following 5 mg/kg amphetamine. When fed a high protein diet, the now heavier mice, had a mean rate of turning of 7.0 ± 0.7 turns/min, while the lighter, low protein diet animals showed a mean turning rate of 10.9 ± 0.8 turns/minute. Spontaneous locomotor activity changed in the same direction as the rate of turning. When the diet pattern was reversed after 3 weeks, the weight differences, spontaneous locomotor activity and rates of turning to amphetamine also reversed. When finally the two groups of mice were fed a normal diet both the weight and amphetamine-induced turning behaviour reverted to the same levels in the two groups.

The results of the biogenic amine and tryptophan levels in forebrains of the high and low protein diet animals are given in Table 1. The different diets caused no significant changes in noradrenaline or dopamine content. There was, however, a significant difference in the 5-hydroxytryptamine and tryptophan levels. The low protein diet caused a highly significant 20% decrease in the level of 5-hydroxytryptamine ($P < 0.001$) and a significant 30% decrease in the level of tryptophan ($P < 0.05$) when compared to the high protein diet mice.

Discussion

This study has used the turning mouse model of von Voigtlander & Moore (1973) to investigate the effect

Table 1 The effect of high and low protein diet on noradrenaline, dopamine, 5-hydroxytryptamine and tryptophan levels in mouse forebrain

Biogenic amine	*High protein diet*	*Low protein diet*	*% difference*	*Significance*
Dopamine	658 ± 47	666 ± 47	1	NS
Noradrenaline	207 ± 11	243 ± 26	15	NS
5-Hydroxy-tryptamine	728 ± 19	578 ± 12	21	$P < 0.001$
Tryptophan	832 ± 82	587 ± 96	30	$P < 0.05$

Biogenic amines expressed as ng/g wet weight of forebrain All observations, mean ± s e. mean, $n = 10$. NS denotes no statistical significance.

of drugs acting on 5-hydroxytryptamine upon amphetamine and apomorphine-induced circling behaviour. This circling phenomenon depends upon an imbalance in activity between the two nigro-striatal dopamine systems which in this instance results from the destruction of dopamine nerve terminals in one striatum by the direct injection of 6-hydroxydopamine. Amphetamine, an agent that releases dopamine from the intact nigro-striatal pathway, causes circling towards the lesioned side, while the dopamine agonist apomorphine causes turning away from the lesioned side presumably because the dopamine receptors on this side have become supersensitive (Ungerstedt, 1971). In this model striatal dopamine levels are reduced on the side of injection: forebrain 5-hydroxytryptamine concentrations are not grossly affected (Pycock *et al.*, 1976). Thus, modification of central 5-hydroxytryptaminergic mechanisms by systemic administration of various drugs would be expected to be effective on both sides of the brain, and the changes observed in circling behaviour probably reflect the action of bilateral changes in 5-hydroxytryptamine on an imbalanced dopamine system.

The rate at which these animals circle to either amphetamine or apomorphine has been shown to be influenced by gross changes in brain 5-hydroxytryptamine levels. Parachlorophenylalanine, an inhibitor of 5-hydroxytryptamine synthesis (Koe & Weissman, 1966), caused an 80% depletion of brain 5-hydroxytryptamine and potentiated the turning induced by amphetamine and, to a lesser extent, that caused by apomorphine. Parachlorophenylalanine has been reported to cause both an increase (Fibiger & Campbell, 1971a) and a decrease (Volicer, 1969) in spontaneous locomotor activity in rats; recently Breese, Cooper & Mueller (1974) have shown that *p*-chlorophenylalanine significantly potentiates amphetamine-induced locomotor activity in rats, although it has no apparent effect on the stereotypies produced by amphetamine. Both our results and those of Breese *et al.* (1974) may be explained by the fact that amphetamine itself may release 5-hydroxytryptamine as well as the catecholamines from nerve endings as suggested by Fuxe & Ungerstedt (1970). Grabowska (1974) also has shown that lesions of the midbrain raphé nuclei which reduce rat forebrain 5-hydroxytryptamine levels by some 38% potentiate apomorphine-induced locomotor activity, while Grabowska *et al.* (1973) have shown that *p*-chlorophenylalanine potentiates and 5-hydroxytryptophan inhibits the stimulating effect of apomorphine on motor behaviour.

Increase in brain 5-hydroxytryptamine levels produced by prior administration of L-tryptophan (Modigh, 1973b) or 5-hydroxytryptophan (Modigh, 1972) is associated with decreased spontaneous locomotor activity (Modigh, 1974). In our experiments these agents decreased the rates of circling induced by both apomorphine and amphetamine. L-Tryptophan increased brain 5-hydroxytryptamine levels by some 50% and decreased apomorphine and amphetamine-induced circling by about 30%. 5-Hydroxytryptophan increased brain 5-hydroxytryptamine levels by 100% and decreased apomorphine and amphetamine-induced circling by 20 and 60% respectively. The differences in the effects of L-tryptophan and 5-hydroxytryptophan on drug-induced circling in relation to their effects on brain 5-hydroxytryptamine are probably due to the relatively non-specific effect of 5-hydroxytryptophan. Whereas L-tryptophan is converted to 5-hydroxytryptamine only in cerebral 5-hydroxytryptaminergic neurones which possess tryptophan hydroxylase (Knapp & Mandell, 1973), 5-hydroxytryptophan is converted into 5-hydroxytryptamine in both 5-hydroxytryptaminergic and catecholaminergic neurones which possess the non-specific L-aromatic amino acid decarboxylase (Butcher, Engel & Fuxe, 1972; Ng, Chase, Colburn & Kopin, 1972), with consequent complex functional effects.

The results of the dietary experiments mimic those obtained with *p*-chlorophenylalanine, 5-hydroxytryptophan and tryptophan. It has been shown that changing dietary tryptophan content causes alterations in brain tryptophan and 5-hydroxytryptamine levels (e.g. Fernstrom & Wurtman, 1972; Biggio, Fadda, Fanni, Tagliamonte & Gessa, 1974). We have confirmed these observations in the present study, when mice fed a high protein diet had both higher brain tryptophan and 5-hydroxytryptamine levels than mice fed a low protein diet. Brain catecholamine levels were not affected. The high protein animals had lower spontaneous locomotor activity than the low protein mice, confirming the observations of Jacobs *et al.* (1974). The animals fed on a high protein diet also turned less vigorously to amphetamine than those fed on a low protein diet, an effect that was convincingly reversed by changing the diet. However perhaps the results obtained from the dietary experiments should be interpreted with some caution, as it has also been shown that food deprivation enhances amphetamine-induced locomotor activity in rats (Fibiger & Campbell, 1971b; Simpson, 1974).

The overall conclusion from these experiments in which brain 5-hydroxytryptamine levels were increased or decreased is that circling behaviour due to striatal dopamine receptor stimulation is depressed by an elevation of brain 5-hydroxytryptamine and enhanced by a reduction in brain 5-hydroxytryptamine. These results are in agreement with those of Baldessarini, Amatruda, Griffith & Gerson (1975) who have demonstrated similar effects on apomorphine-induced turning in rats with unilateral electrolytic lesions of the nigro-striatal pathway.

The results obtained with drugs believed to block or stimulate central 5-hydroxytryptamine receptors were less clear. Methysergide, a drug claimed to block 5-hydroxytryptamine receptors (Bieger, Larochelle & Hornykiewicz, 1972), did not cause any consistent or dose-dependent effect on either apomorphine or amphetamine-induced circling behaviour. However there is doubt as to how well this drug penetrates into the brain (Doepfner, 1962), although it enhances apomorphine-induced locomotor activity (Grabowska & Michaluk, 1974) and stereotypy (Weiner, Goetz & Klawans, 1975) and apomorphine-induced turning in rats (Baldessarini *et al.*, 1975).

Similarly, no conclusive effect was demonstrated with LSD. Although LSD was originally described as an agent that blocks cerebral 5-hydroxytryptamine receptors (Boakes, Bradley, Briggs & Dray, 1970) and inhibits the release of this transmitter from central neurones (Chase, Breese & Kopin, 1967), later evidence suggests that it may stimulate 5-hydroxytryptamine receptors (Andén, Corrodi, Fuxe & Hökfelt, 1968; Aghajanian, 1972). Further, it has now been suggested that LSD is also a potent dopamine receptor agonist, for it causes strong contraversive turning in rats with unilateral lesions of the medial forebrain bundle (Pieri, Pieri & Haefely, 1974). However, no such effect was found in the present study, where only nigro-striatal terminals were damaged by the unilateral 6-hydroxydopamine lesion (Pycock & Anlezark, 1975). Pieri *et al.*'s (1974) result may have been due to the fact that their lesion affected pathways other than the nigro-striatal dopaminergic tract; particularly as Costall & Naylor (1974) have shown that a unilateral electrolytic lesion in the 5-hydroxytryptaminergic raphé nuclear system will result in a rat that circles in response to drugs. Furthermore, Grabowska *et al.* (1974) found that LSD had little effect on apomorphine-induced locomotor activity in rats, suggesting that it does not possess potent dopamine agonist action.

In contrast to methysergide and LSD, cyproheptadine, another anti-5-hydroxytryptamine agent (Stone, Wenger, Luddon, Stavorski & Ross, 1961), did cause dose-dependent inhibition of both amphetamine and apomorphine-induced circling. This result is the reverse of that expected on the basis of the earlier experiments. 5-Hydroxytryptamine receptor blockade should have mimicked the effects of brain 5-hydroxytryptamine depletion which caused an increase in drug-induced circling. We have no ready explanation for this finding, but it may be due to other unrecognized pharmacological actions of cyproheptadine, which is known, for example, to possess antihistaminic properties (Stone *et al.*, 1961).

The effect of the antidepressant drug, clomipramine, was investigated because it is a potent inhibitor of 5-hydroxytryptamine uptake into cerebral 5-hydroxytryptaminergic neurones (Carlsson, Corrodi, Fuxe & Hökfelt, 1969), with only a weak effect on the uptake of noradrenaline and dopamine into catecholamine neurones (Shaskan & Snyder, 1970, Fuck & Punell, 1973). Clomipramine might have been expected to enhance cerebral 5-hydroxytryptamine transmission, and therefore to depress circling induced by amphetamine. In fact, in doses of between 1.25 and 10 mg/kg, it caused a small increase in the rate of circling produced by a subthreshold dose of amphetamine. This may have been due to its albeit weak capacity to block reuptake of dopamine, for drugs such as nomifensine which are powerful dopamine reuptake blockers, do potentiate amphetamine-induced circling in this mouse model (Pycock, Milson, Tarsy & Marsden, unpublished observations). Also the inconsistency in the results may be explained by an inhibition of the rate at which amphetamine is metabolized similar to that seen after other tricyclic compounds as described by Lewander (1969).

The question arises as to the site at which 5-hydroxytryptamine impinges on the nigro-striatal dopamine system. Anatomical and biochemical evidence suggests that 5-hydroxytryptamine neurones enter both the substantia nigra and the striatum. Thus synaptosomes prepared from the substantia nigra contain large quantities of 5-hydroxytryptamine (Parizek, Hassler & Bak, 1971) which is consistent with the concept that there is a 5-hydroxytryptamine neurone projection to the substantia nigra (Andén, Dahlström, Fuxe, Larsson, Olson & Ungerstedt, 1966). Similarly, the large quantities of 5-hydroxytryptamine in the striatum probably lie mainly in the nerve terminals of 5-hydroxytryptamine neurones arising from the midbrain raphé nuclei (Andén *et al.*, 1966; Poirier, Singh, Boucher, Bouvier, Olivier & Larochelle, 1967; Kim, Hassler, Kurokawa & Bak, 1970). Stimulation of the raphé nuclei in cats results in release of 5-hydroxytryptamine from the caudate nucleus (Ashkenazi, Holman & Vogt, 1972; Holman & Vogt, 1972), while anatomical studies have identified a pathway from the anterior raphé nuclei to the basal ganglia in cats (Brodal, Taber & Walberg, 1960).

It is possible that such 5-hydroxytryptamine neurones influence the firing of nigro-striatal dopamine fibres themselves, or alternatively they could affect the activity of those nerve cells in the striatum controlled by incoming dopamine nigro-striatal fibres. The experiments undertaken in this study might have given some indication as to which of these two possibilities is the most likely. If 5-hydroxytryptamine manipulation affected only circling induced by amphetamine, an action directly on the nigro-striatal dopamine system would seem most plausible. On the other hand if in our model both apomorphine and amphetamine-induced circling was equally affected, then an action on striatal neurones would be most probable. In fact, the experiments did not give such a clear-cut answer. The exact electrophysiological inter-relation of basal ganglia dopamine and 5-hydroxytryptamine pathways remains to be established by further experiment.

C.P. is a fellow of the Parkinson's Disease Society. J.M. gratefully acknowledges support from Ciba-Geigy Ltd., and the King's College Hospital Research Fund. Mr Colin Brewer provided expert technical assistance. The authors wish to thank Professor C.D. Marsden for his valuable comments on the manuscript, and Smith, Kline and French Laboratories Ltd., for gifts of dexamphetamine sulphate.

References

AGHAJANIAN, G.K. (1972). LSD and CNS transmission. *Ann. Rev. Pharmac.*, **12**, 157–168.

ANDÉN, N.-E., CORRODI, H., FUXE, K. & HÖKFELT, T. (1968). Evidence for a central 5-hydroxytryptamine receptor stimulation by lysergic acid diethylamide. *Br. J. Pharmac.*, **34**, 1–7.

ANDÉN, N.-E., DAHLSTRÖM, A., FUXE, K. & LARSSON, K. (1966). Functional role of the nigro-neostriatal dopamine neurons. *Acta pharmac. toxicol.*, **24**, 263–274.

ANDÉN, N.-E, DAHLSTRÖM, A., FUXE, K, LARSSON, K., OLSON, L. & UNGERSTEDT, U. (1966). Ascending monoamine neurons to the telencephalon and diencephalon. *Acta physiol. scand.*, **67**, 313–326.

ASHKENAZI, R., HOLMAN, R.B. & VOGT, M. (1972). Release of transmitters on stimulation of the nucleus linearis raphé in the cat. *J. Physiol.*, **233**, 255–259.

BAK, I.J, CHOI, W.B., HASSLER, R., USUNOFF, K.G. & WAGNER, A. (1975). Fine structural synaptic organisation of the corpus striatum and substantia nigra in rat and cat. In *Adv. Neurol.*, **9**, 25–41, ed. Calne, D.B., Chase, T.N. & Barbeau, A., New York: Raven Press.

BALDESSARINI, R.J., AMATRUDA, T.T., GRIFFITH, F.F. & GERSON, S. (1975). Differential effects of serotonin on turning and stereotypy induced by apomorphine. *Brain Res.*, **93**, 158–163.

BESSON, M.J, CHERAMY, A., FELTZ, P & GLOWINSKI, J. (1969). Release of newly synthesized dopamine from dopamine-contining terminals in the striatum of the rat. *Proc. Nat. Acad. Sci., U.S.A.*, **62**, 741–748.

BIEGER, D., LAROCHELLE, L. & HORNYKIEWICZ, O. (1972). A model for the quantitative study of central dopaminergic and serotoninergic activity. *Eur. J. Pharmac.*, **18**, 128–136.

BIGGIO, G., FADDA, F., FANNI, P., TAGLIAMONTE, A. & GESSA, G.L. (1974). Rapid depletion of serum tryptophan, brain trytophan, serotonin and 5-hydroxyindole acetic acid by a tryptophan-free diet. *Life Sciences*, **14**, 1321–1329.

BOAKES, R.J, BRADLEY, P.B., BRIGGS, I. & DRAY, A. (1970). Antagonism of 5-hydroxytryptamine by LSD 25 in the central nervous system; a possible neuronal basis for the actions of LSD 25. *Br. J. Pharmac*, **40**, 202–218.

BREESE, G.R., COOPER, B.R. & MUELLER, R.A. (1974). Evidence for involvement of 5-hydroxytryptamine in the actions of amphetamine. *Br. J. Pharmac.*, **52**, 307–314.

BRODAL, A, TABER, E & WALBERG, F. (1960). The raphé nuclei of the brain stem in the cat. II. Efferent connections. *J. comp. Neurol.*, **114**, 239–260.

BUTCHER, L L., ENGEL, J. & FUXE, K. (1972). Behavioural, biochemical, and histochemical analyses of the central effects of monoamine precursors after peripheral decarboxylase inhibition. *Brain Res.*, **41**, 387–411.

CARLSSON, A., CORRODI, H., FUXE, K. & HÖKFELT, T. (1969). Effect of antidepressant drugs on the depletion of intraneuronal brain 5-hydroxytryptamine stores caused by 4-methyl-α-ethyl-meta-tyramine. *Eur. J. Pharmac.*, **5**, 357–366.

CHANG, C.C. (1964). A sensitive method for spectrophotofluorometric assay of catecholamines. *Int. J. Neuropharmac.*, **3**, 643–649.

CHASE, T.N., BREESE, G.R. & KOPIN, I.J. (1967). Serotonin release from brain slices by electrical stimulation: regional differences and effect of LSD. *Science*, **157**, 1461–1463.

COSTALL, B & NAYLOR, R.J (1974). Stereotyped and circling behaviour induced by dopaminergic agonists after lesions of the midbrain raphé nuclei. *Eur. J. Pharmac.*, **29**, 206–222.

DENCKLA, W.D. & DEWEY, H.K. (1967). The determination of tryptophan in plasma liver and urine. *J. Lab. Clin. Med.*, **69**, 160–168.

DOEPFNER, W. (1962). Biochemical observations on LSD—25 and Deseril. *Experientia*, **18**, 256–257.

FERNSTROM, J.D. & WURTMAN, R.J. (1972). Brain serotonin content: physiological regulation by plasma neutral amino acids. *Science*, **178**, 414–416.

FIBIGER, H.C. & CAMPBELL, B.A. (1971a). The effect of parachlorophenylalanine on spontaneous locomotor activity in the rat. *Neuropharmac.*, **10**, 25–32.

FIBIGER, H.C. & CAMPBELL, B.A. (1971b). Potentiation of amphetamine-induced arousal by starvation. *Nature, Lond.*, **233**, 424–425.

FUXE, K., HÖKFELT, T. & UNGERSTEDT, U. (1968). Localization of indolealkylamines in CNS. *Adv. Pharmac.*, **6**, 235–251.

FUXE, K. & UNGERSTEDT, U. (1970). Histochemical, biochemical and functional studies on central monoamine neurons after acute and chronic amphetamine administration. In *Amphetamines and Related Compounds*, ed. Costa, E. & Garattini, S. pp 257–288. New York: Raven Press.

GRABOWSKA, M. (1974). Influence of midbrain raphé lesions on some pharmacological and biochemical effects of apomorphine in rats. *Psychopharmac., Berl.*, **39**, 315–322.

GRABOWSKA, M., ANTKIEWICZ, L., MAJ, J. & MICHALUK, J. (1973). Apomorphine and central serotonin neurones. *Pol. J. Pharmac. Pharm.*, **25**, 29–39.

GRABOWSKA, M. & MICHALUK, J. (1974). On the role of serotonin in apomorphine-induced locomotor stimulation in rats. *Pharmac. Biochem. Behav.*, **2**, 263–266.

HADŽOVIĆ, S. & ERNST, A.M. (1969). The effect of 5-hydroxytryptamine and 5-hydroxytryptophan on extrapyramidal function. *Eur. J. Pharmac.*, **6**, 90–95.

HOLMAN, R.B. & VOGT, M. (1972). Release of 5-hydroxytryptamine from caudate nucleus and septum. *J. Physiol.*, **223**, 243–254.

JACOBS, B.L. (1974). Effect of two dopamine receptor blockers on a serotonin-mediated behavioural syndrome in rats. *Eur. J. Pharmac.*, **27**, 363–366.

JACOBS, B.L., EUBANKS, E.E. & WISE, W.D. (1974). Effect of indolealkylamine manipulations on locomotor activity in rats. *Neuropharmac.*, **13**, 575–583.

KIM, J.S., HASSLER, R., KUROKAWA, M. & BAK, I.J. (1970). Abnormal movements and rigidity induced by harmaline in relation to striatal acetylcholine, serotonin and dopamine. *Expl. Neurol.*, **29**, 189–200.

KNAPP, S. & MANDELL, A.J. (1973). Some drug effects on the functions of the two measurable forms of tryptophan-5-hydroxylase: influence on hydroxylation and uptake of substrate. In *Serotonin and Behaviour*, ed. Barchas, J. & Usdin, E. pp 61–71. New York: Academic Press.

KOE, B.K. & WEISSMAN, A. (1966). *p*-Chlorophenylalanine: a specific depletor of brain serotonin. *J. Pharmac. exp. Ther.*, **154**, 499–516.

LAVERTY, R. & SHARMAN, D.F. (1965). The estimation of small quantities of 3,4-dihydroxyphenylethylamine in tissues. *Br. J. Pharmac. Chemother.*, **24**, 538–548.

LEWANDER, T. (1969). Influence of various psychoactive drugs on the *in vivo* metabolism of d-amphetamine in the rat. *Eur. J. Pharmac.*, **6**, 38–44.

MAICKEL, R.P., COX, R.H., SAILLANT, J. & MILLER, F.P. (1968). A method for the determination of serotonin and norepinephrine in discrete areas of rat brain. *Int. J. Neuropharmac.*, **7**, 275–281.

MODIGH, K. (1972). Central and peripheral effects of 5-hydroxytryptophan on motor activity in mice. *Psychopharmac., Berl.*, **23**, 48–54.

MODIGH, K. (1973a). Effects of isolation and fighting in mice on the rate of synthesis of noradrenaline, dopamine and 5-hydroxytryptamine in the brain. *Psychopharmac., Berl.*, **33**, 1–17.

MODIGH, K. (1973b). Effects of L-tryptophan on motor activity in mice. *Psychopharmac., Berl.*, **30**, 123–134.

MODIGH, K (1974). Functional aspects of 5-hydroxytryptamine turnover in the central nervous system. *Acta physiol. scand., Suppl.*, **403**, 1–56.

NG, L.K.Y., CHASE, T.N., COLBURN, R.W. & KOPIN, I.J. (1972). Release of ^{3}H-dopamine by L-5-hydroxytryptophan. *Brain Res.*, **45**, 499–505.

PARIZEK, J., HASSLER, R. & BAK, I.J. (1971). Light and electromicroscopic autoradiography of substantia nigra of rat after intra-ventricular administration of tritium labelled norepinephrine, dopamine, serotonin and the precursors. *Z. Zellforsch. mikrosk. Anat.*, **115**, 137–148.

PIERI, L., PIERI, M. & HAEFELY, W. (1974). LSD as an agonist of dopamine receptors in the striatum. *Nature, Lond.*, **252**, 586–588.

POIRIER, L.J., SINGH, P., BOUCHER, R., BOUVIER, G., OLIVIER, A. & LAROCHELLE, P. (1967). Effect of brain lesions on striatal monoamines in the cat. *Arch. Neurol.*, **17**, 601–608.

PYCOCK, C. & ANLEZARK, G. (1975). LSD and dopamine receptors. *Nature, Lond.*, **257**, 69–70.

PYCOCK, C., TARSY, D. & MARSDEN, C.D. (1976). Inhibition of circling behaviour by neuroleptic drugs in mice with unilateral 6-hydroxydopamine lesions of the striatum. *Psychopharmac.* (in press).

SHASKAN, E.G. & SNYDER, S.H. (1970). Kinetics of serotonin accumulation into slices from rat brain: relationship to catecholamine uptake. *J. Pharmac. exp. Ther.*, **175**, 404–418.

SIMPSON, L.L. (1974). A study of the interaction between amphetamine and food deprivation. *Psychopharmac., Berl.*, **38**, 279–286.

STONE, C.L., WENGER, H.C., LUDDEN, C.T., STRAVORSKI, J.M. & ROSS, C.A. (1961). Antiserotonin-antihistaminic properties of cyproheptadine. *J. Pharmac. exp. Ther.*, **131**, 73–84.

TUCK, J R. & PUNELL, G. (1973). Uptake of ^{3}H-5-hydroxytryptamine and ^{3}H-noradrenaline by slices of rat brain incubated in plasma from patients treated with chlorimipramine, imipramine or amitriptyline. *J. Pharm. Pharmac.*, **25**, 573–574.

UNGERSTEDT, U (1971). Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of the nigro striatal dopamine system. *Acta physiol. Scand.*, **82**, *Suppl.* **367**, 69–93.

VON VOIGTLANDER, P.F. & MOORE, K.E. (1973). Turning behaviour in mice with unilateral 6-hydroxydopamine lesions in the striatum: effects of apomorphine, L-DOPA, amantadine, amphetamine and other psychomotor stimulants. *Neuropharmac.*, **12**, 451–462.

VOLICER, L. (1969). Correlation between behavioural and biochemical effects of *p*-chlorophenylalanine in mice and rats. *Int. J. Neuropharmac.*, **8**, 361–364.

WEINER, W.J., GOETZ, C. & KLAWANS, H.L. (1975). Serotonergic and antiserotonergic influences on apomorphine-induced stereotyped behaviour. *Acta pharmac. toxicol.*, **36**, 155–160.

WEINER, W.J., GOETZ, C., WESTHEIMER, R. & KLAWANS, H.L. (1973). Serotonergic and antiserotonergic influences on amphetamine-induced stereotyped behaviour. *J. neurol. Sciences*, **20**, 373–379.

(Received June 23, 1975
Revised August 16, 1975)

Br. J. Pharmac. (1976), **56**, 87–91

THE EFFECT OF LITHIUM CHLORIDE ON ONE-TRIAL PASSIVE AVOIDANCE LEARNING IN RATS

F.N. JOHNSON
Department of Psychology, University of Lancaster, Fylde College, Bailrigg, Lancaster

1 Expression of a one-trial passive avoidance learning response in rats was examined following injections of lithium chloride or sodium chloride before and after initial training and before the first day of testing. Five tests were given at daily intervals, 24 h after training being the time of the first test.

2 Lithium given before the first day of testing impaired response expression on the first and all subsequent days of testing; the rate of extinction was unaffected.

3 Given both before and immediately after initial training, lithium impaired response expression on the first day of testing but slowed down the subsequent rate of extinction, leading eventually to improved performance on the fifth day, as compared with placebo-treated control subjects.

4 The results are interpreted in the light of the hypothesis that lithium impaired the central processing of sensory information.

Introduction

In recent years an increasing number of reports has appeared indicating that lithium salts may produce behavioural effects in animals (see reviews by Johnson, 1972, 1975a, 1975b). Whilst it is still too early for the results of such studies to be reliably related to the therapeutic effects which lithium is known to have in conditions of manic-depression (Gershon & Shopsin, 1973) the findings are intriguing enough to warrant further investigation. Attention has recently been directed to the effects which lithium ions may have upon the establishment of memory traces immediately after a learning experience: the results of several studies in this area have led to conflicting interpretations.

Mark & Watts (1971) and Watts & Mark (1971) showed that when lithium chloride was injected directly into the forebrain of day-old chicks 5 min before they were trained in a one-trial passive avoidance learning situation, the retention of subsequent avoidance responses was impaired. The investigators related this effect to a lithium-induced inhibition of short-term memory traces. The same test situation was subsequently used by Benowitz & Sperry (1973) but with rather different results: retention of avoidance responding seemed to be less impaired when testing was done 20 min after training than when the training-test interval was as long as 24 hours. Since, on the basis of many studies involving electroschock- and drug-induced modification of learning and memory (e.g. McGaugh & Madsen, 1964; Kumar, Stolerman & Steinberg, 1970), short-term memory traces may be regarded as being largely replaced by consolidated long-term traces by 20 min after learning, these results seemed to indicate that lithium effects, if they were indeed upon memory traces of any kind, were probably not upon the short-term type. Benowitz & Sperry (1973) therefore suggested that the lithium effect might be upon a behaviourally inactive, convert type of trace, distinct from the short-term trace, and which functioned as a necessary precursor of long-term memory; this new type of trace had first been proposed by Benowitz & Magnus (1973).

Johnson & Barker (1972) reported effects of lithium chloride on escape-avoidance learning in rats, which they related to a possible drug action on processes interfering with short-term memory consolidation: the experiments described below produced findings which can be similarly interpreted and which may provide the basis for an alternative explanation of the results of the Benowitz & Sperry (1973) study.

Methods

Thirty male rats of the Roman Control strain were used in the present investigations. All rats were aged approximately 100 days at the start of the experiment. They were housed 3 to a cage and provided with water and food *ad libitum.*

Apparatus

The apparatus consisted of a shelf, 10 cm front to back × 23 cm wide, elevated 10 cm above a 25 cm square floor comprising parallel metal bars through which a scrambled 0.25 mA a.c. (50 Hz) constant current electric shock could be administered to the animals' feet for a period of 5 seconds. A guillotine door could be lowered in front of the shelf to restrain animals either on the shelf or on the shock bars. The whole apparatus was bounded by black Perspex walls 30 cm high and topped by a clear Perspex hinged lid. Room fluorescent lighting was used throughout.

Procedure

On day 1 each subject was placed upon the shelf, the guillotine door raised after a 10 s delay, and the time taken for the animal to step down and place all four feet on the shock bars was recorded with a hand-operated stop-watch. The guillotine door was then lowered to prevent the rat returning to the shelf. Shocks were delivered to the rat's feet immediately after the door had been lowered. The subject was then removed by hand from the apparatus and returned to its home cage. The step-down latency was again measured, in the same apparatus, at 24 h intervals, on days 2 to 6. A cut-off criterion of 10 min was established but was not in fact needed. Shocks were not given in the test sessions and passive avoidance responses acquired on day 1 were thus allowed to undergo extinction.

Subject selection

All rats showing step-down latencies of less than 5 s or more than 30 s on the initial training trial on day 1 were rejected and replaced by others. In all, 3 rats were rejected on these criteria in choosing the 30 experimental animals.

Injections

All drug injections of either lithium chloride or placebo (sodium chloride), at dose levels of 2.5 mmol/kg body weight, were made intraperitoneally, 0.1 ml of drug or placebo solution being injected for each 100 g subject body weight. Drug solutions were coded, the code being broken by the experimenter only after the experiment had been completed.

Experimental design

Two drug conditions, lithium chloride and placebo (sodium chloride) were used, injections being made at one of three times relative to the onset of the training shock: 10 min before the shock, 15 s after the shock, or 10 min before the first training trial on Day 2 (i.e. 23 h 50 min after the shock). Five rats, all conforming to the initial selection criteria noted previously, were examined in each of the drug/injection-time conditions. The experimental design was thus a 2 (drugs) × 3 (injection times) × 5 (extinction tests) factorial, with 5 subjects per condition and repeated measures on the third factor.

Results

The findings are shown in Figure 1. Over all there was found to be a clear reduction in response (step-down) latency over the five test trials, $F(d.f.\ 4{,}96)=31.53$; $P<0.001$, indicating that the avoidance response acquired on the training session underwent progressive extinction throughout the series of test sessions. However, the picture was complicated by the presence of a statistically significant effect of injection time, $F(d.f.\ 2{,}24)=3.76$; $P<0.05$, and a drug × extinction test interaction, $F(d.f.\ 4{,}96)=3.72$; $P<0.01$, though the three-way interaction between drugs, injection-times and extinction tests just failed to attain statistical significance at the $P=0.05$ level.

Reference to Figure 1 reveals that whilst the major extinction trend was visible under most experimental conditions it failed to occur to such a marked degree in those animals given lithium chloride injections either before, or immediately after, the training session: in these two cases the animals showed considerably greater retention of avoidance learning on the fifth test session than was exhibited by the corresponding placebo-treated control subjects or by animals given either lithium or placebo before the first test trial. In the animals injected with lithium chloride 10 min before the first test, avoidance responding was reduced on the first and on all four subsequent tests. A separate analysis of variance carried out on the pre-test injection data indicated that there was a significant over-all drug effect $F(d.f.\ 2{,}7)=4.80$; $P<0.05$, but the drug × extinction test interaction failed to attain statistical significance at the $P=0.05$ level. In all lithium-treated subjects, irrespective of injection time, avoidance responding on the first test session was less marked than that exhibited by placebo-treated control animals, the effects at the three injection times being assessed as significant at the $P=0.05$ level using the Newman-Keuls procedure for making specific comparisons between means (Winer, 1962).

Discussion

Pre-testing injection effects

Johnson (1972, 1975b) has suggested that many of the effects elicited by lithium ions in behavioural test

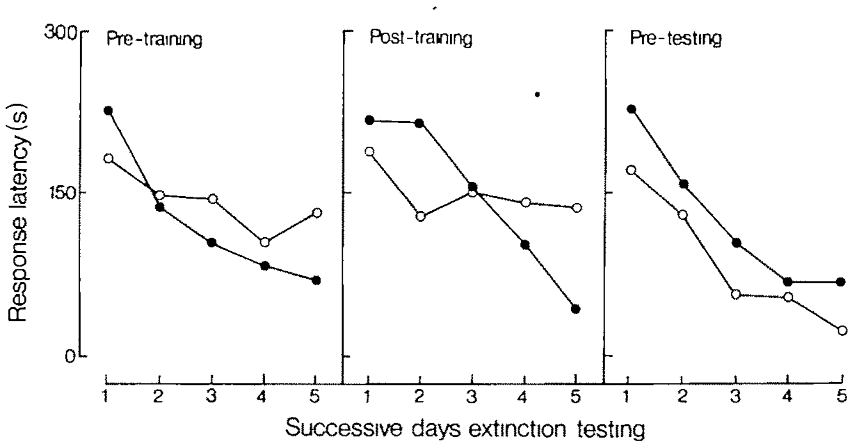


Figure 1 Injections of lithium chloride (O) before and after one-trial passive avoidance training caused impairment of avoidance responding on the first day of testing, but subsequent extinction over the next four test sessions was less rapid than that shown by control subjects. Pre-testing injections led to reduced avoidance responding on all days of testing (●) = Placebo

situations may be explained on the basis of a lithium-induced impairment of the efficiency with which an animal analyses and attaches significance, or importance, to sensory input. It was argued that even if such an impairment were only slight, affecting sensory information around threshold levels, this might be sufficient to be reflected in behavioural changes in appropriately designed experiments. On this hypothesis, lithium chloride given before testing might be expected to impair avoidance performance by effectively preventing an animal from processing all the environmental cues needed to elicit the learned response. This would lead to a shortened step-down latency on the first day of testing, as observed in the present experiment.

The reduced latencies occurring on the subsequent four test sessions cannot similarly be explained unless it is assumed that lithium ions are still present in the animal's tissues following the single injection before the first test trial: this seems unlikely. It may be that the early step-down on the first occasion leads to an enhanced extinction effect which is then reflected in subsequent trials and maintained throughout the test series.

Pre- and post-training effects

The explanation of the effects produced by lithium chloride injections given 10 min before, or 15 s after, training on day 1, is not to be found in the drug's effects on the test procedures. In the first place, as previously noted, it is unlikely that much lithium remains unexcreted 24 h after administration, and secondly the effects of peri-training administration differ quite markedly in form from those of pre-testing injection. We must examine the process of response acquisition and retention to elucidate the mechanisms of lithium action.

Only the pre-training injections can affect acquisition processes, but both pre- and post-training injections can operate upon the processes of memory trace establishment and consolidation, provided that the lithium ions are not entirely eliminated from the body and are still able to affect neural processes 10 min after injection and into the immediate post-training period. In view of the similarities between the effects of pre- and post-training lithium injections (see Figure 1) it seems reasonable and parsimonious to relate them to the same mechanisms. Figure 2 illustrates what these mechanisms might be.

Short-term memory traces (stm) are regarded as being established during the training period, subsequently decaying rapidly in the post-training period. Meanwhile, more stable long-term memory traces (ltm) consolidate in the post-training period. Such a model is certainly an oversimplification as far as human memory is concerned (Norman, 1970) but it will suffice for the description of most animal studies on memory formation.

Lithium chloride, it is suggested, produces its effects noted in the present studies by exerting its influence during the period of short-term memory decay and long-term memory consolidation. Two separate effects may be involved.

Firstly, it is well documented (Kumar *et al.*, 1970) that many central nervous depressant drugs may interfere with memory establishment and it has been suggested that this may result from the impairment of

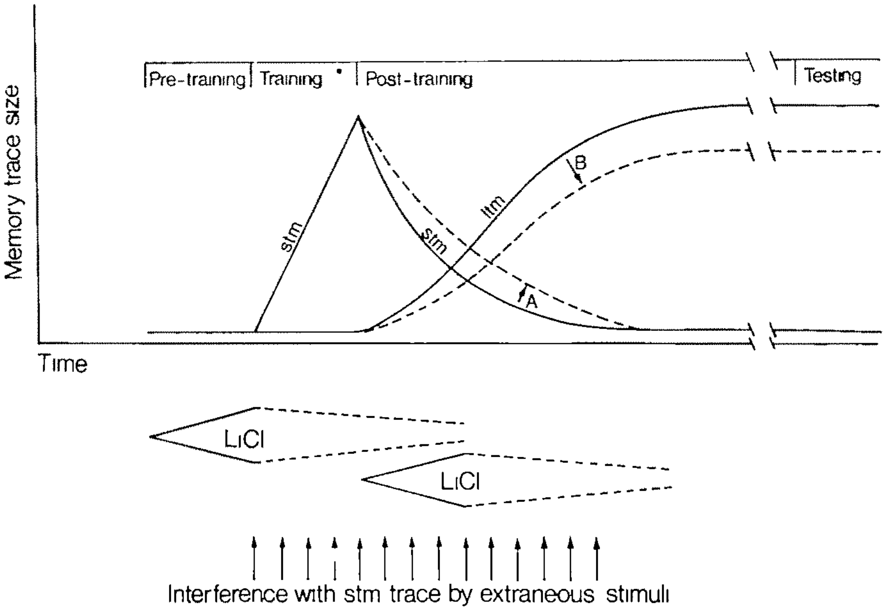


Figure 2 Schematic representation of the hypothesized effects of lithium on memory traces stm: short-term memory trace; ltm: long-term memory trace; LiCl: effective lithium chloride concentrations after pre- or post-training injection, the pre-training injection continuing to give rise to effective tissue concentrations into the post-training period; A: reduced stm trace decay rate as a result of LiCl blockade of interfering stimuli; B. impaired consolidation of ltm leading to a smaller final ltm trace size.

ltm consolidation. Whilst lithium may not fit entirely happily into a central nervous depressant classification it does nevertheless have some properties characteristic of such drugs and it may be that it has an effect on ltm consolidation (B in Figure 2). This would lead to the formation of a smaller ltm trace and consequently to less marked expression of avoidance learning on the first test trial, as observed in the experiment.

The reduced rate of extinction of the avoidance response, as assessed over the full five-day period, which follows pre- and post-training lithium chloride administration may be related to an effect of the drug upon the decay rate of the stm trace. If an animal is responsive to stimuli arising from its environment in the immediate post-shock period (and it is possible that shock administration leads to heightened awareness and hyperresponsiveness) then it might be that at least part of the stm decay process is due to interference between the trace and new memory traces which arise just after the learning experience but which are irrelevant to it. By blocking, or impairing, the analysis of these extraneous stimuli, as it has been suggested that lithium chloride might do (Johnson, 1972, 1975b), lithium ions could prevent the formation of the interfering traces and delay the stm decay process (A in Figure 2).

Johnson (1970) has suggested that the parameters of stm decay rate may in some way be encoded in the ltm trace so as to determine the stability, assessed as the resistance to extinction, of the latter. In other words, the ltm trace, although reduced in size by the lithium treatment given in association with the learning experience is, at the same time, made more stable and relatively refractory to modification by the extinction process. A similar explanation has been used to account for chlorpromazine effects on memory, chlorpromazine being a drug which, in its therapeutic profile, may have some similarities with lithium salts (Johnson, 1971a, b).

It is possible to use the interpretation suggested above to explain the effects observed by Benowitz & Sperry (1973). If lithium chloride does indeed extend the duration of the stm trace this might still be available, or available to an enhanced degree, 20 min after the learning experience to contribute to the expression of avoidance responding, thus producing a better avoidance response than that observed 24 h post-learning when only the ltm trace remains to be behaviourally active.

Alternative explanations of the findings are also possible. If, in the experiments of Benowitz & Sperry (1973), lithium chloride was still available 20 min after administration, but not 24 h later, the finding of little impairment of learning at the earlier test time may be related to a state-dependence effect (Overton, 1966) assuming that the lithium was active during learning acquisition as well as in the post-training period.

Secondly, it is still not known whether lithium ions are entirely eliminated 24 h after administration (Fyrö & Sedvall, 1975) and if there were some residual effect of any remaining drug during the testing procedure this might account for the impaired performance on the first test occasion. The lowered rate of extinction could then be related to enhanced ltm formation following protection against stm interference in the manner previously suggested.

This work was carried out while the author held a lectureship in the Department of Psychology, University of Birmingham, England, and thanks are due to Professor P.L. Broadhurst for providing the animal and experimental facilities. I am grateful to Dr T.J. Crow for pointing out the alternative ways of explaining these results, described in the last two paragraphs of the Discussion section.

References

BENOWITZ, L & MAGNUS, J.G. (1973). Memory storage processes following one-trial aversive conditioning in the chick. *Behav. Biol.*, **8**, 121–129.

BENOWITZ, L & SPERRY, R.W. (1973). Amnesic effects of lithium chloride in chicks. *Exp. Neurol.*, **40**, 540–546.

FYRÖ, B. & SEDVALL, G. (1975). The excretion of lithium. In *Lithium research and therapy*, ed. Johnson, F.N., pp. 287–312. London: Academic Press.

GERSHON, S & SHOPSIN, B. (1973). *Lithium: its role in psychiatric research and treatment.* New York: Plenum Press.

JOHNSON, F N. (1970). The use of the Kamin effect to distinguish between size and stability characteristics of long-term memory traces. *Life Sci.*, **9**, 1175–1179.

JOHNSON, F.N. (1971a). Stimulus significance and chlorpromazine-induced impairment of avoidance learning in mice. *Neuropharmacology*, **10**, 9–14.

JOHNSON, F.N. (1971b). Stimulus significance and chlorpromazine effects on the expression of avoidance learning in mice. *Neuropharmacology*, **10**, 267–272.

JOHNSON, F.N (1972). Chlorpromazine and lithium: effects on stimulus significance. *Dis. nerv. Syst.*, **33**, 235–241.

JOHNSON, F.N. (1975a). Behavioural and cognitive effects of lithium: observations and experiments. In *Lithium research and therapy*, ed. Johnson, F.N., pp. 315–337. London: Academic Press.

JOHNSON, F.N. (1975b). Behavioural and cognitive effects of lithium: mechanisms. In *Lithium research and therapy*, ed. Johnson, F.N., pp. 339–350. London: Academic Press.

JOHNSON, F.N. & BARKER, J.G. (1972). Effects of lithium chloride on learned responses: acquisition, retention, expression. *Dis. nerv. Syst.*, **33**, 664–666.

KUMAR, R., STOLERMAN, I.P. & STEINBERG, H. (1970). Psychopharmacology. *Ann. Rev. Psychol.*, **21**, 595–628.

MARK, R.F. & WATTS, M.E. (1971). Drug inhibition of memory formation in chickens: I. Long-term memory. *Proc. R. Soc. B.*, **178**, 439–454.

McGAUGH, J.L. & MADSEN, M.C. (1964). Amnesic and punishing effects of electroconvulsive shock. *Science*, **144**, 182–183.

NORMAN, D A. (1970). *Models of human memory.* New York: Academic Press.

OVERTON, D.A. (1966). State-dependent learning produced by depressant and atropine-like drugs. *Psychopharmacologia*, **10**, 6–31.

WATTS, M.E. & MARK, R.F. (1971). Drug inhibition of memory formation in chickens: II. Short-term memory. *Proc. R. Soc. B.*, **178**, 455–464.

WINER, B.J. (1962). *Statistical principles in experimental design.* New York: McGraw-Hill.

(Received June 27, 1975.
Revised August 15, 1975)

Br. J. Pharmac. (1976), **56**, 93–99

THE EFFECT OF EDROPHONIUM ON ERYTHROCYTE ACETYLCHOLINESTERASE AND NEUROMUSCULAR FUNCTION IN THE RAT

H.E. BARBER, T.N. CALVEY, K.T. MUIR & K. TAYLOR
Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX

1 The relation between the concentration of edrophonium in plasma, inhibition of red cell acetylcholinesterase, and neuromuscular transmission was studied in the rat.

2 In both *in vivo* and *in vitro* conditions, red cell acetylcholinesterase activity was predictably related to the concentration of the quaternary amine.

3 After low doses of edrophonium (1.0 µmol/kg) there was a significant correlation between the monophasic potentiation of twitch tension and the plasma concentration of the drug. In contrast, with higher doses of edrophonium (4.0 or 10.0 µmol/kg) a biphasic potentiation of twitch tension was observed; this was only correlated with the plasma concentration of the drug during the secondary decline in neuromuscular facilitation. Subsequent recovery of normal neuromuscular transmission invariably occurred at a constant plasma concentration of edrophonium.

Introduction

It has been recognized for many years that edrophonium chloride has a rapid and transient effect on neuromuscular transmission (Randall, 1950). Nevertheless, the mechanism of action of this phenolic quaternary amine on the motor end-plate has not been precisely defined. Edrophonium reversibly inhibits acetylcholinesterase (acetylcholine acetylhydrolase; EC 3.1.1.7) and has a direct action on the motor end-plate (Randall, 1950; Wescoe & Riker, 1951), although these effects may play only a subsidiary role in the facilitation of neuromuscular transmission. For instance, it has been suggested that the drug acts by inducing antidromic discharges in presynaptic terminals that are disseminated by local axon reflexes (Blaber, 1972). In these conditions, the pharmacological effects of edrophonium may not be predictably related to its plasma concentration or to acetylcholinesterase inhibition. Previous experiments have shown that the unchanged drug is rapidly removed from the circulation (Back & Calvey, 1972b); the relation between its plasma concentration and neuromuscular transmission remains a matter of conjecture.

This paper is concerned with two main aspects of this problem. In the first place, we have studied the relation between the concentration of edrophonium in plasma and the inhibition of acetylcholinesterase activity in rat erythrocytes. These experiments have been carried out in both *in vivo* and *in vitro* conditions. Secondly, the relation between the clearance of edrophonium from plasma, the facilitation of neuromuscular transmission, and the subsequent recovery of normal twitch tension has been investigated. A preliminary account of this work has been presented to the British Pharmacological Society (Barber, Calvey, Muir & Taylor, 1975).

Methods

In vitro *studies*

Male Wistar rats (250–300 g) were anaesthetized with ethyl carbamate (14% w/v in distilled water; 1.4 g/kg, i.p.) and a carotid artery was cannulated with polyethylene tubing. Heparin sodium (approximately 200 u in 0.2 ml) was injected into the arterial cannula and the animals were then exsanguinated.

In most experiments, aqueous solutions of edrophonium chloride (1 µmol/l, 2 µmol/l, 5 µmol/l, 10 µmol/l, 20 µmol/l, 50 µmol/l, 100 µmol/l, 200 µmol/l, 500 µmol/l, 1 mmol/l; 10 µl) were added to rat blood (1.0 ml). A capillary tube was filled with a sample of blood (about 50 µl), sealed at one end, and centrifuged (12,000 *g*; 2 minutes). The concentration of edrophonium in plasma was calculated from the haematocrit value. It was assumed that edrophonium was solely distributed in plasma, and that the quaternary amine was not bound to (or did not penetrate) the red cell membrane. The validity of this assumption was confirmed by concurrent studies with [^{14}C]-edrophonium (ethyl[1-^{14}C]-dimethyl (3-hydroxyphenyl)-ammonium chloride, specific radioactivity = 13.7 mCi/mmol; The Radiochemical Centre, Amersham, Bucks). In these experiments, [^{14}C]-edrophonium chloride (100 nCi in 10 µl) was added to rat blood (1.0 ml) and the concentration of the drug in plasma was determined by liquid scintillation spectrometry. Similar results were obtained in both types of experiments.

In vivo *studies*

Male Wistar rats (250–350 g) were anaesthetized with ethyl carbamate (1.4 g/kg i.p.) and a polyethylene cannula was placed in a jugular vein. Respiration was assisted (when necessary) by means of an endotracheal tube and a Palmer miniature respiration pump (C.F. Palmer Ltd., Sandwich, Kent). The contraction of the tibialis anterior muscle in response to supramaximal stimulation of the sciatic nerve (0.33 Hz, 0.5 ms) was measured from both hind limbs by means of a strain gauge. Arterial blood pressure was recorded from a common carotid artery using a polyethylene cannula filled with heparin sodium (approximately 100 u/ml) which was attached to a pressure transducer.

The contralateral common carotid artery was also cannulated and heparin sodium (200 u in 0.2 ml) was injected intravenously at the beginning of each experiment.

[^{14}C]-edrophonium chloride (1.0, 4.0, or 10.0 µmol/kg in 0.9% w/v NaCl solution) was injected into the jugular vein within 5 seconds. Samples of arterial blood (approximately 250 µl) were removed at 1, 2, 3, 5, 10, 15, 30, 45, 60, 90, 120 and 180 minutes. Small amounts of heparin sodium (1000 u/ml) were occasionally used to prevent coagulation in the carotid arterial cannula between the collection of consecutive blood samples. Plasma was obtained from part of each sample by centrifugation, and the remainder of the sample was used for the assay of acetylcholinesterase activity.

Measurement of [^{14}C]-edrophonium in plasma

Samples of plasma (usually 50 µl) were directly added to scintillation fluid (9 ml) of the following composition: butyl-PBD (6.7 mg), toluene (5.0 ml), Triton-X-100 (3.0 ml) and distilled water (1.0 ml). A mixture of isoamyl alcohol and toluene (1:5, v/v; 1.0 ml) was then added to each scintillation vial. Radioactivity in plasma was counted at an efficiency of approximately 60% in a Unilux Nuclear Chicago liquid scintillation spectrometer. Counting efficiency was determined by the channels ratio method; similar efficiencies (±1%) were obtained when [1-^{14}C]-*n*-hexadecane (The Radiochemical Centre) was used as an internal standard.

Unchanged [^{14}C]-edrophonium was separated from its glucuronide metabolite in plasma by descending paper chromatography (Back & Calvey, 1972a). Radioactivity on paper chromatograms was identified in a Tracerlab 4π radiochromatogram scanner. The concentration of [^{14}C]-edrophonium was then determined from the total radioactivity in plasma and the results of paper chromatography.

Measurement of acetylcholinesterase activity in blood cells

Acetylcholinesterase activity in red blood cells was measured by a modification of the method of Smith (1974). Samples of [1-^{14}C]-acetyl-β-methylcholine iodide (specific radioactivity = 9.92 mCi/mmol; NEN Chemicals GmbH; 25.4 nmol in 50 µl sodium phosphate buffer, (40 mmol/l; pH = 7.4)) were placed in glass vials and freeze dried. All measurements of acetylcholinesterase activity were carried out in a cold room at 4°C.

Immediately prior to the assay, blood specimens were thoroughly stirred in a vortex mixer and pre-cooled in an ice-cold water bath. Blood (50 µl) was added to freeze-dried [^{14}C]-acetyl-β-methylcholine; after 1 min, the reaction was stopped by the addition of HCl (100 µl; 200 mmol/litre). [1-^{14}C]-acetic acid was extracted from the mixture as described by Smith (1974), and radioactivity was counted by liquid scintillation spectrometry.

Results

In vitro *studies*

There was a sigmoid relationship between the concentration of edrophonium in plasma and the activity of red cell acetylcholinesterase (Figure 1). When the concentration of the quaternary amine in rat plasma was increased from 0.1 µmol/l to 2.0 µmol/l, acetylcholinesterase activity decreased from 80% to 10% of control values. Between these limits, there was a linear relationship between the logarithm of edrophonium concentration and acetylcholinesterase activity (Figure 1). When the concentration of the drug was 10.0 µmol/l or greater, no enzyme activity was present in rat erythrocytes.

In vivo *studies*

After intravenous injection of [^{14}C]-edrophonium the plasma concentration of the unchanged drug rapidly decreased within 10–15 min (Table 1; Figure 2). The rapid decline in the concentration of the drug was seen

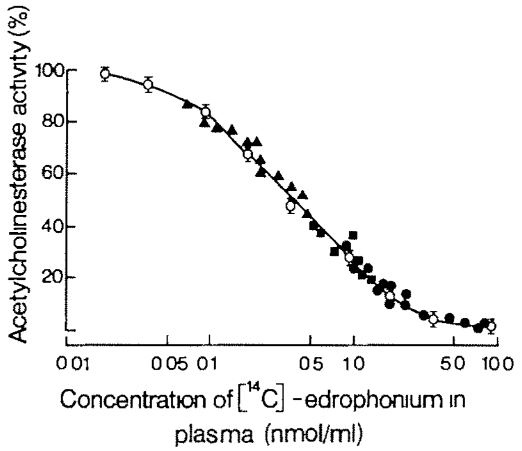


Figure 1 Effect of edrophonium on red cell acetylcholinesterase activity. Each open circle and vertical bar represents the mean ± s.d of at least 3 observations in *in vitro* conditions. Closed symbols correspond to results obtained in *in vivo* conditions at three different dose levels: (●) 10.0 μmol/kg; (■) 4.0 μmol/kg; (▲) 1.0 μmol/kg.

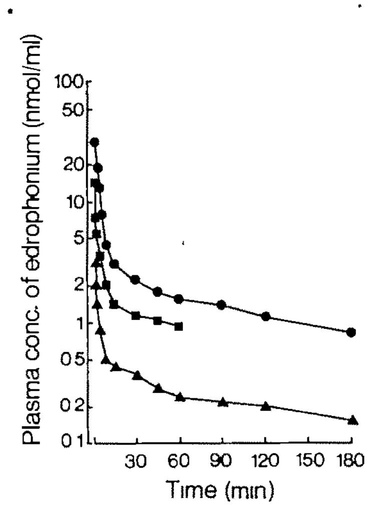


Figure 2 The concentration of unchanged [^{14}C]-edrophonium in plasma after intravenous administration of three different doses of the drug: (●) 10.0 μmol/kg; (■) 4.0 μmol/kg; (▲) 1.0 μmol/kg. Each graph represents the results of a typical experiment.

at all three dose levels. After 10 min, the plasma concentration of unchanged drug decreased more slowly and a semi-logarithmic plot relating edrophonium concentration to time (Figure 2) could be invariably resolved into at least two exponential components by the method of residuals (Riggs, 1963).

Measured concentrations of [^{14}C]-edrophonium in plasma were related to red cell acetylcholinesterase activity (Figure 1). Three different doses of the drug were used in order to compare *in vitro* and *in vivo* enzyme inhibition. After intravenous injection of edrophonium (10 μmol/kg) acetylcholinesterase activity gradually increased to 31% of control values (Figure 1). Corresponding enhancement of enzyme activity was associated with the lower doses of edrophonium. There was a statistically significant correlation between acetylcholinesterase inhibition in *in vivo* and *in vitro* conditions ($r=0.99$, $d.f.=89$; $P<0.001$; Figure 1).

Table 1 The concentration of unchanged [^{14}C]-edrophonium in plasma after intravenous administration of three different dose levels (1.0 μmol/kg, 4.0 μmol/kg and 10.0 μmol/kg)

	Dose of [^{14}C]-edrophonium		
	1.0 μmol/kg	*4.0 μmol/kg*	*10.0 μmol/kg*
Time (min)	*Plasma concentration of edrophonium nmol/ml*	*nmol/ml*	*nmol/ml*
1	2.97±0.09 (4)	10.90±1.39 (3)	29.67±1.86 (3)
2	2.03±0.09 (4)	6.35±0.64 (4)	17.77±2.41 (3)
3	1.43±0.08 (4)	4.78±0.40 (4)	12.70±0.89 (3)
5	0.85±0.04 (4)	3.33±0.16 (4)	8.20±1.37 (3)
10	0.44±0.01 (4)	2.06±0.30 (4)	4.93±0.44 (3)
15	0.33±0.04 (4)	1.41±0.04 (4)	3.60±0.25 (3)
30	0.26±0.04 (4)	0.96±0.09 (4)	2.30±0.00 (3)
45	0.25±0.02 (4)	0.75±0.10 (4)	1.84±0.03 (3)
60	0.19±0.02 (4)	0.65±0.10 (4)	1.63±0.03 (3)
90	0.15±0.05 (4)		1.43±0.07 (3)
120	0.12±0.03 (4)		1.15±0.04 (3)
180	0.09±0.02 (4)		0.96±0.02 (3)

Data are means ± s.e. mean, with the number of experiments in parentheses

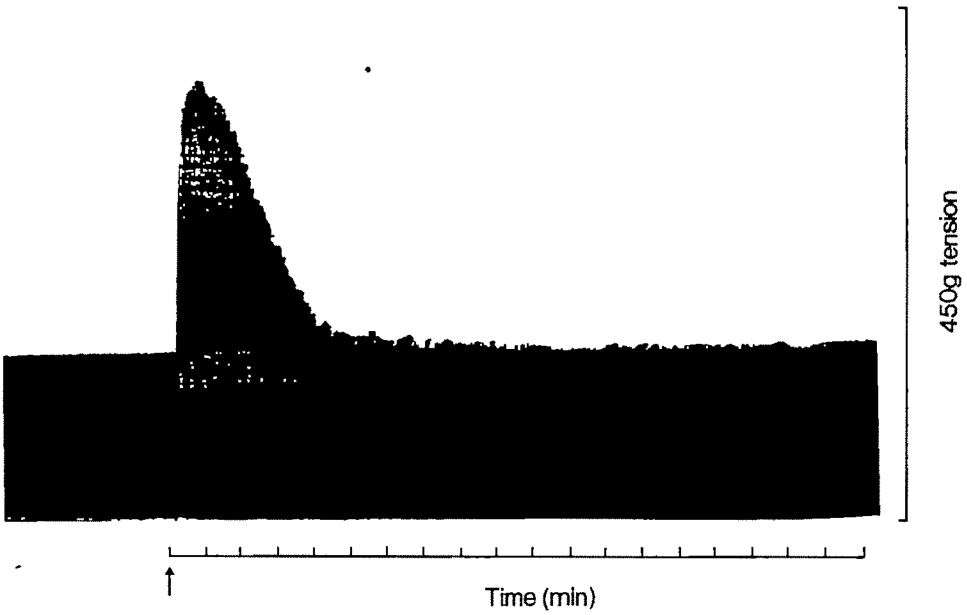


Figure 3 Effect of edrophonium (1.0 μmol/kg) on contraction of tibialis anterior muscle in response to supramaximal stimulation of the sciatic nerve (0.33 Hz, 0.5 ms). Figure retouched

Relation between plasma concentration of edrophonium and facilitation of neuromuscular transmission

After intravenous injection of edrophonium (1.0 μmol/kg), neuromuscular transmission was facilitated in a monophasic manner. Tibialis twitch tension rapidly increased, but returned to its control value within 5 min (Figure 3). The maximum plasma concentration of edrophonium (2.97 ± 0.09 nmol/ml; Table 1) was associated with an increase in twitch tension to at least twice control values (Figure 4). Twitch tension returned to its control value after 5 min at a plasma concentration of 1.32–1.80 nmol/ml. There was a statistically significant correlation between the concentration of edrophonium in plasma and facilitation of neuromuscular transmission, as assessed by the percentage increase in control twitch tension ($r = 0.87$, $d.f. = 14$; $P < 0.001$; Figure 4).

Different results were obtained after intravenous administration of higher doses of edrophonium (4.0 μmol/kg or 10.0 μmol/kg). In these conditions, neuromuscular transmission was enhanced biphasically (Figure 5). An immediate, evanescent increase in twitch tension rapidly declined, and was succeeded by a second facilitatory phase that decreased to control levels within 15 min (Figure 5). When the experimental data preceding the peak of the second facilitatory phase were considered, there was no significant correlation between the concentration of edrophonium in plasma and the percentage change in twitch tension, at either 4.0 μmol/kg ($r = -0.36$, $d.f. = 9$, $P > 0.05$) or 10.0 μmol/kg ($r = -0.12$, $d.f. = 12$, $P > 0.05$). A wide range of plasma concentrations of edrophonium

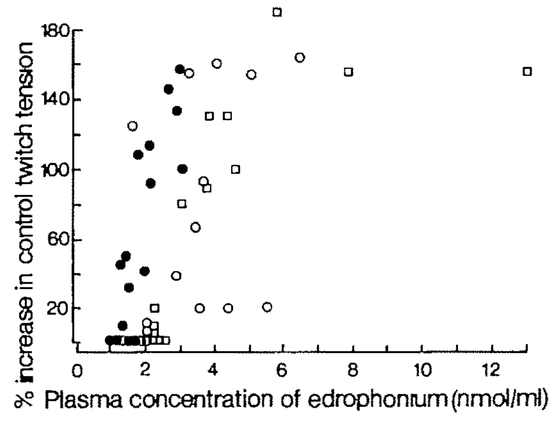


Figure 4 Correlation between the plasma concentration of edrophonium and the percentage increase in twitch tension of the tibialis muscle compared with control values. Dose of edrophonium = 1.0 μmol/kg (●), 4.0 μmol/kg (○) or 10.0 μmol/kg (□). At the higher dose levels (4.0 or 10.0 μmol/kg), results obtained before the peak of the second phase of neuromuscular facilitation were omitted.

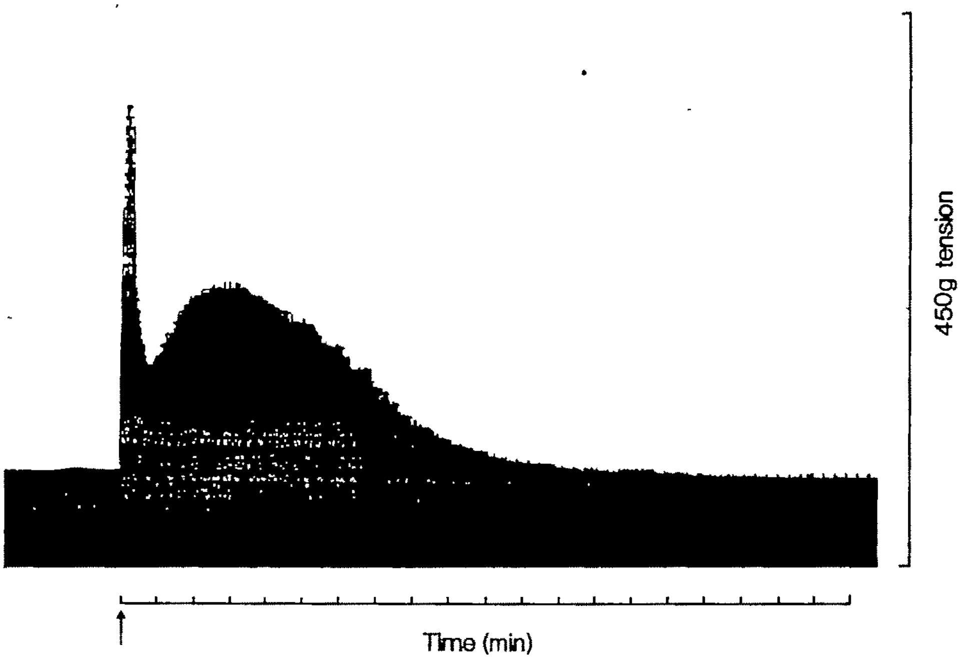


Figure 5 Effect of edrophonium (4.0 μmol/kg) on contraction of tibialis anterior muscle in response to supramaximal stimulation of the sciatic nerve (0.33 Hz, 0.5 ms). Figure retouched.

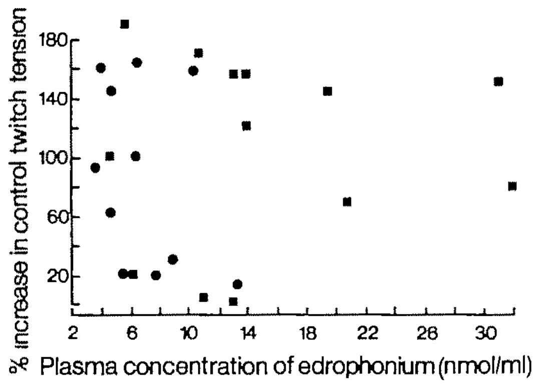


Figure 6 Relation between the plasma concentration of edrophonium and the percentage increase in twitch tension over control values before the peak of the second phase of neuromuscular facilitation. Dose of edrophonium =4.0 μmol/kg (●) or 10.0 μmol/kg (■).

(4.1–31.0 nmol/ml) was associated with enhancement of the tibialis twitch tension to more than twice its control value (Figure 6). In contrast, the decline in tibialis twitch tension from its peak value during the second facilitatory phase (Figure 5) was significantly correlated with the clearance of the drug from plasma at both 4.0 μmol/kg ($r=0.53$, $d.f.=15$; $0.05>P>0.01$) and 10.0 μmol/kg ($r=0.73$, $d.f.=12$; $0.01>P>0.001$) (Figure 4). The plasma concentration at which tibialis twitch tension returned to normal was remarkably constant (1.97 ± 0.12 nmol/ml; mean ± s.e. mean).

Discussion

Since edrophonium chloride combines reversibly with cholinesterase (Wilson, 1955), the activity of the enzyme should be predictably related to the concentration of the quaternary amine. Unfortunately, many of the available methods used to determine cholinesterase activity (for instance, techniques involving dilution or significant concentrations of high affinity substrates) cannot be reliably applied to the assay of reversible enzyme inhibitors. Radiometric determinations of acetylcholinesterase activity (Smith, 1974) are less subject to these limitations, since they depend on the use of relatively small concentrations of lyophilized, low affinity substrate. These methods have been used in the present experiments; although some reversal of enzyme inhibition must occur on addition of the substrate, measurements of acetylcholinesterase activity should be closely related to the concentration of edrophonium in both *in vivo* and *in vitro* conditions.

The results of this study are entirely consistent with this concept. There was a sigmoid relationship between the logarithm of plasma edrophonium concentration and acetylcholinesterase activity in *in vitro* conditions. At plasma concentrations of 0.1–2.0 nmol/ml, the relation between these parameters was linear. In addition, when [^{14}C]-edrophonium was injected intravenously, and the measured concentration of the drug in plasma was related to acetylcholinesterase activity, a similar relationship was observed. The results of these experiments have two important implications. In the first place, since acetylcholinesterase activity is directly related to the plasma concentration of edrophonium in both *in vitro* and *in vivo* conditions, the relation between the two parameters can be used to measure the plasma concentration of the quaternary amine. Secondly, the present results confirm that the glucuronide metabolite of edrophonium (ethyldimethyl-(3-oxyphenyl) ammonium glucuronide; Back & Calvey, 1972a) has little or no biological activity. If, for instance, the metabolite inhibited acetylcholinesterase to any significant extent, the relationship between the concentration of edrophonium in plasma and enzyme activity would differ in *in vivo* and *in vitro* conditions.

After three different doses of edrophonium the plasma concentration of the unchanged drug decreased in a qualitatively similar manner. An initial rapid fall was followed by a slower declining phase, and a semi-logarithmic plot relating plasma concentration to time could be resolved into more than one exponential component. Similar results were obtained in an earlier study (Back & Calvey, 1972b) in which the clearance of a single dose of edrophonium was studied in the rat. The present experiments were carried out at three different dose levels; in general, the results are consistent with the rapid removal of edrophonium from plasma, irrespective of the dose.

The effects of edrophonium on neuromuscular transmission have been studied by many authors (Randall, 1950; Hobbiger, 1952; Katz & Thesleff, 1957; Blaber & Bowman, 1959; Ferry & Marshall, 1971; Blaber, 1972; Whittaker, 1975). In spite of the use of sophisticated electrophysiological techniques, its precise mechanism of action on the neuromuscular junction has not been unequivocally established. Studies on the isolated tenuissimus muscle of the cat suggest that edrophonium produces antidromic discharges in presynaptic nerve terminals. These antidromic impulses are spread by axon reflexes to other nerve terminals, resulting in repetitive end-plate potentials (Blaber, 1972). Other authors consider that their experimental data can be best explained in terms of the action of the drug on end-plate acetylcholinesterase. Thus in the rat isolated diaphragm the prolongation of the end-plate potential is due to inhibition of the enzyme (Ferry & Marshall, 1971). Similarly, in *in vivo* conditions the effect of edrophonium on miniature end-plate potentials, end-plate potentials, and the quantal release of transmitter suggest that acetylcholinesterase inhibition is probably responsible for the facilitation of neuromuscular transmission (Whittaker, 1975). In the present work, we have been primarily concerned with the relation between the pharmacological action of the drug and its clearance from plasma. After low doses of edrophonium, there was a statistically significant correlation between the potentiation of twitch tension and the plasma level of the drug. A similar relationship was seen during the recovery from facilitation induced by high doses of edrophonium; thus the concentration of the drug in plasma at which normal neuromuscular function returned was constant, irrespective of the dose of the quaternary amine. Since the plasma level was directly related to erythrocyte acetylcholinesterase activity, these results are not apparently inconsistent with the classical view that reversible inhibition of the enzyme is responsible for the pharmacological action of the drug at the neuromuscular junction (Hobbiger, 1952; Katz & Thesleff, 1957; Ferry & Marshall, 1971; Whittaker, 1975). However, it cannot be necessarily assumed that acetylcholinesterase activity in red blood cells and at the neuromuscular junction are closely related. If edrophonium acts on presynaptic nerve terminals (Blaber, 1972), the high concentrations of acetylcholine released locally may rapidly reverse enzyme inhibition at the motor end-plate but not on the red cell membrane. In these conditions, inhibition of erythrocyte cholinesterase may not be directly related to enzyme activity or other presynaptic and postsynaptic events at the neuromuscular junction. Thus, the significance of the correlation between the potentiation of tibialis twitch tension and red cell acetylcholinesterase activity is a matter of conjecture. It is, perhaps, more likely that both enzyme activity and the pharmacological effects of edrophonium at the motor end-plate are independently correlated with the plasma level of the drug.

In contrast, the rapid increase in twitch tension induced by large doses of edrophonium, and its subsequent decline, were quite independent of the plasma level of the drug or red cell acetylcholinesterase inhibition. In these circumstances, the explanation for the biphasic response to edrophonium is quite obscure, although the initial facilitation may be followed by the onset of 'receptor inactivation' (Hubbard, 1973). In *in vitro* conditions, the amplitude of extracellular end-plate potentials is increased by low concentrations of edrophonium, although opposite effects are produced by high concentrations of the drug (Ferry & Marshall, 1971). The results of the present experiments are not inconsistent with these concepts, although they do not unequivocally exclude other mechanisms of action.

The authors are grateful to Dr G.A. Buckley for helpful discussions, and the technical assistance of Miss S. Newby is gratefully acknowledged. This research was supported by grants from the Peel Medical Research Trust and the Muscular Dystrophy Group of Great Britain. Correspondence to T.N.C.

References

BACK, D.J. & CALVEY, T.N. (1972a). Excretion of ^{14}C-edrophonium and its metabolites in bile: role of the liver cell and the peribiliary vascular plexus. *Br. J. Pharmac.*, **44**, 534–543.

BACK, D.J. & CALVEY, T.N. (1972b). The removal of ^{14}C-edrophonium from the circulation. *Br. J. Pharmac.*, **46**, 355–357.

BARBER, H.E., CALVEY, T.N., MUIR, K.T. & TAYLOR, K (1975). The relation between the plasma concentration of edrophonium, inhibition of erythrocyte acetylcholinesterase, and the facilitation of neuromuscular function in the rat. *Br. J. Pharmac.*, **54**, 263P.

BLABER, L.C. (1972). The mechanism of the facilitatory action of edrophonium in cat skeletal muscle. *Br. J. Pharmac.*, **46**, 498–507.

BLABER, L.C. & BOWMAN, W.C (1959). A comparison between the effects of edrophonium and choline in the skeletal muscles of the cat. *Br. J. Pharmac.*, **14**, 456–466.

FERRY, C.B. & MARSHALL, A.R. (1971). Electrophysiological estimation of inhibition of acetylcholinesterase with normal transmitter release. *J. Physiol.*, **218**, 66–67P.

HOBBIGER, F. (1952). The mechanism of anticurare action of certain neostigmine analogues. *Br. J. Pharmac. Chemother.*, **7**, 223–236.

HUBBARD, J.I (1973). Microphysiology of vertebrate neuromuscular transmission. *Physiol. Rev.*, **53**, 674–723.

KATZ, B. & THESLEFF, S (1957). The interaction between edrophonium (Tensilon) and acetylcholine at the motor end-plate. *Br. J. Pharmac. Chemother.*, **12**, 260–264.

RANDALL, L.O. (1950). Anticurare action of phenolic quaternary ammonium salts. *J. Pharmac. exp. Ther.*, **100**, 83–93.

RIGGS, D.S. (1963). *The mathematical approach to physiological problems*, pp. 146–161. Baltimore: The Williams and Wilkins Company.

SMITH, M.H. (1974). Determination of blood acetylcholinesterase inhibition by reversible inhibitors *in vivo*. *Biochem. Med.*, **10**, 236–244.

WESCOE, W.C. & RIKER, W.F. (1951). The pharmacology of anticurare agents. *Ann. N.Y. Acad. Sci.*, **54**, 438–455.

WHITTAKER, R. (1975). Neuromuscular actions of edrophonium in the lateral segmental tail muscle of the rat *in vivo*. *J. Pharm. Pharmac.*, **27**, 420–424.

WILSON, I.B. (1955). The interaction of tensilon and neostigmine with acetylcholinesterase. *Arch. int. Pharmacodyn. Thér.*, **104**, 204–213.

(Received July 3, 1975)

Br. J. Pharmac. (1976), **56**, 101–110

RESPONSES OF NEURONES IN THE CEREBRAL CORTEX AND CAUDATE NUCLEUS TO AMANTADINE, AMPHETAMINE AND DOPAMINE

T.W. STONE

Department of Physiology, University of Aberdeen, Marischal College, Aberdeen, Scotland

1 Dopamine, amantadine and amphetamine have been applied directly by microiontophoresis to single neurones in the caudate nucleus and cerebral cortex of rats anaesthetized with urethane.

2 The predominant response to all three agents was a depression of neuronal firing rate. The responses to dopamine and amantadine could be antagonized by the dopamine receptor blocking agent, chlorpromazine.

3 Amantadine did not cause any potentiation of dopamine responses, suggesting that inhibition of amine uptake was not responsible for its effects.

4 The responses of pyramidal tract cells in the cerebral cortex to dopamine, amphetamine and amantadine were compared in control groups of rats and rats pretreated with reserpine (10 mg/kg i.p.) or α-methyl-p-tyrosine methyl ester (200 mg/kg i.p.). The reduction of cortical catecholamine concentrations was confirmed by a direct fluorimetric assay method.

5 Responses to dopamine were unaltered in the amine-depleted animals compared with controls. Responses to amantadine and amphetamine were reduced but not abolished.

6 It is concluded that amantadine acts partly by releasing catecholamines from neuronal stores. The residual responses to amantadine and amphetamine may be the result of a direct postsynaptic receptor stimulation.

Introduction

Amantadine hydrochloride (1-amino-adamantan; Symmetrel) is being used increasingly in the treatment of Parkinson's disease (Birdwood, Gilder & Wink, 1971). This use has developed from the observations of Schwab, England, Poskanzer & Young (1969) that amantadine, which was for several years used as an antiviral agent, could substantially reduce Parkinsonian symptoms.

The mechanism of amantadine's anti-Parkinsonian action is not completely understood. The behavioural effects of amantadine, such as motor hyperactivity (Lassen, 1973; Maj, Sowińska & Baran, 1973), stereotypy (Strömberg & Svensson, 1971; Cox & Tha, 1973), and turning in unilaterally striatotomized rats (Strömberg & Svensson, 1971) suggest a stimulation of central catecholamine receptors. The turning behaviour in particular indicates that amantadine can activate the nigrostriatal dopaminergic neurone system (Andén, Dahlström, Fuxe & Larsson, 1966; Arbuthnott & Crow, 1971; Crow, 1971). Since it is a disturbance of this system which appears to be a crucial factor in the aetiology of Parkinsonism (Hornykiewicz, 1966; Calne, 1970) it has been assumed that it is amantadine's ability to act on this system which underlies its anti-Parkinsonian properties.

The literature contains evidence that amantadine may act by directly stimulating post-synaptic receptors (Cashin & Sutton, 1973; Lassen, 1973; Papeschi, 1974), by releasing catecholamines (Grelak, Clark, Stump & Vernier, 1970; Scatton, Cheramy, Besson & Glowinski, 1970; Farnebo, Fuxe, Goldstein, Hamberger & Ungerstedt, 1971; Spilker & Dhasmana, 1974), or by inhibiting the neuronal uptake of catecholamines (Fletcher & Redfern, 1970; Baldessarini, Lipinski & Chace, 1972; Heimans, Rand & Fennessy, 1972).

The present experiments were designed to investigate the effects of amantadine on neurones in the caudate nucleus and cerebral cortex of rats when applied by microiontophoresis.

The effects of amantadine were compared with responses to dopamine itself and to amphetamine, which is thought to stimulate central catecholamine receptors indirectly by releasing the amines from neuronal storage sites (Moore, 1963; Carlsson, Lindqvist, Dahlström, Fuxe & Masuoka, 1965; Carlsson, Lindqvist, Fuxe & Hamberger, 1966;

Christie & Crow, 1971; Boakes, Bradley & Candy, 1972). Chlorpromazine was used as a dopamine receptor blocking agent (van Rossum, 1966; York, 1972) to examine the specificity of amantadine's effects on dopamine receptors.

Neuronal responses were also examined in rats pretreated with reserpine or the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine methyl ester, both of which deplete the central nervous system of catecholamines.

Some of these results have been communicated to the Pharmacological Society (Stone, 1975).

Methods

Forty male hooded Wistar rats weighing 250–300 g were used in this study. Fourteen of these were used for the qualitative examination of cortical and caudate neurone responses, and 26 were divided into control and test groups for the later amine-depletion experiments. For all acute experiments, animals were anaesthetized with urethane 1–1.25 g/kg intraperitoneally. The lower doses were needed for amine-depleted rats, which died within 1 h if given the normal dose of 1.25 g/kg. The anaesthetic was sufficient to produce full surgical anaesthesia in all animals.

The left fronto-parietal cerebral cortex was exposed. The animals' rectal temperatures were automatically maintained at 37°–38°C by means of a heating pad and rectal thermistor probe (Krnjević & Mitchell, 1961).

A stereotaxic frame was used which allowed orientation of the head suitable for using the stereotaxic atlas of Fifková & Marsala (1967). Penetrations into the caudate nucleus were made at co-ordinates AP 0, L 3.0, V 5.0.

Five-barrelled micropipettes were used for microiontophoresis, each barrel having a resistance of 2–12 MΩ when filled with 3 M KCl. The pipettes contained one or more of the following solutions: acetylcholine chloride 200 mM, pH 4.5 (Koch-Light); dopamine hydrochloride, 200 mM, pH 4.0 (Koch-Light); (+)-amphetamine sulphate 200 mM, pH 4.5 (S.K. & F.); amantadine hydrochloride 200 mM, pH 4.5 (Geigy); sodium L-glutamate 200 mM, pH 6.5 (B.D.H.); 5-hydroxytryptamine creatinine sulphate 50 mM, pH 4.5 (Koch-Light); chlorpromazine hydrochloride 200 mM, pH 4.5 (May & Baker).

Micropipettes also contained 200 mM sodium chloride in one barrel so that current balancing and current testing was possible (Stone, 1972a). Unit activity was recorded through a single microelectrode containing molar potassium acetate or chloride, fixed alongside the iontophoretic complex (Stone, 1973b). This allowed more satisfactory recording of small spike activity, and allowed all five barrels of the multi-barrel assembly to be used for microiontophoresis. Spikes were amplified by a Fenlow AD 55 preamplifier and either displayed directly on Telequipment oscilloscopes, or passed through a capacitance coupled circuit to filter out slow waves, then through a spike height discriminator and an Ecko Ratemeter. A continuous reading of neuronal firing rate was obtained on a Servoscribe pen recorder.

Depletion of central catecholamines

To examine the effects of amantadine and amphetamine in animals whose brains were depleted of catecholamines, four groups of animals were studied. One group (8 rats) was injected 24 h before the acute experiment with reserpine (10 mg/kg i.p.) suspended in Tween 80. A control group (8 rats) received Tween 80 (1 ml/kg i.p.). A third group (4 rats) was treated 24 h before iontophoresis with α-methyl-p-tyrosine methyl ester hydrochloride (AMPT) (200 mg/kg i.p.) in 0.9% w/v NaCl solution (saline) and the corresponding control animals (6 rats) received saline (1 ml/kg i.p.).

Estimation of catecholamine concentrations

To confirm that reserpine and AMPT did reduce central catecholamine concentrations, the brains of most of the experimental animals were perfused with saline via a carotid artery at the end of the acute experiment and the frontal and parietal cerebral cortices were removed and stored at –28°C. The cortices were subsequently homogenized and the noradrenaline and dopamine concentrations estimated by the method of Cox & Perhach (1973). The method involved conversion of the catecholamines to fluorescent derivatives, the concentrations of which were measured in an Aminco-Bowman SPF 125 spectrofluorimeter. The excitation and emission wavelengths used were 320 nm and 370 nm (dopamine) and 385 nm and 485 nm (noradrenaline).

Comparison of neuronal responses

To try and reduce the variation of neuronal responses normally found with microiontophoresis, the cells used for comparing amine responses in normal and amine-depleted animals in the present study were all spontaneously active, identified pyramidal tract cells. The identification of these cells has been described previously (Stone, 1972b).

To enable any differences to be detected in the neuronal responses of normal and amine-depleted animals it was necessary to measure various parameters of the neuronal response, which could then be subjected to statistical analysis. Three parameters were measured: h, the maximum change of firing rate produced; t, the time between beginning a drug ejection and h; d, the total duration of response. All three measurements were made with respect to a line drawn through the basal firing rate existing before the drug ejection. Also, to facilitate comparison of the results, all the agonist compounds were ejected with currents of 60 nA for 15 seconds.

Results

All the units studied in the cerebral cortex and 50% of those in the caudate nucleus were spontaneously active. The remaining 50% of caudate units were excited artificially by iontophoretic pulses of glutamate (Connor, 1970) (60 nA for 5 s every 30 s) or acetylcholine (60 nA for 15 s periods).

Qualitative examination of responses of caudate and cortical units

In the initial experiments the three agonists, dopamine, amantadine and amphetamine were each ejected with outward currents of 60 nA. A summary of the results obtained, including number of cells tested, the type of response and the changes of firing rate produced is presented in Table 1.

Examples of records of neuronal firing rates are shown in Figure 1. Figure 1a is taken from a spontaneously active cortical neurone. Figure 1b and 1c, showing firing rates in the caudate nucleus, illustrates that depression by the agonists could be seen when cells were excited with glutamate (Figure 1b) or acetylcholine (Figure 1c). Nevertheless if a cell was depressed by any of the agonists, the firing

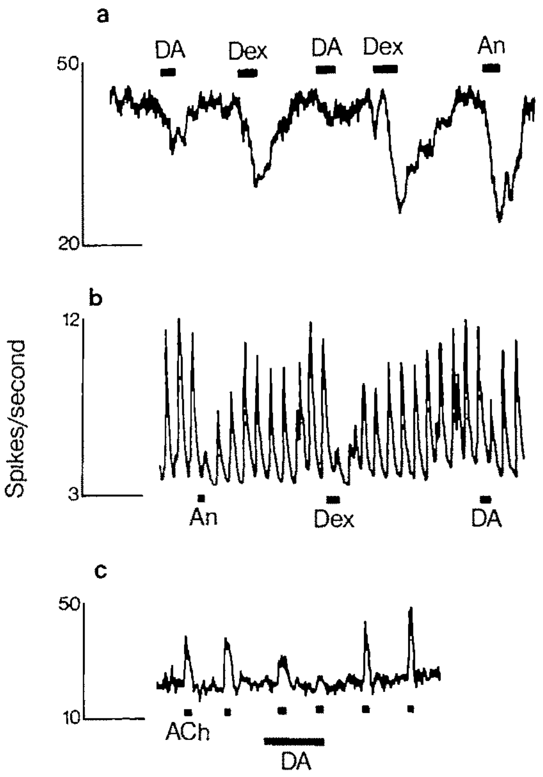


Figure 1 Records of the firing rates of neurones showing. (a) a cortical unit which is depressed by the iontophoresis of dopamine, 60 nA (DA), amphetamine, 60 nA (Dex), and amantadine, 60 nA (An) Note the apparently greater potency of the latter two drugs (b) A neurone in the caudate nucleus which showed little spontaneous activity and which was therefore excited by applications of glutamate (60 nA for 5 s every 30 s). Depression of the cell excitability is thus revealed as a reduction in the height of the glutamate response. (c) A neurone in the caudate nucleus which was spontaneously active, but which was further excited by acetylcholine, 60 nA (ACh) applied as indicated by bars beneath record Depression of acetylcholine responses by dopamine shows that dopamine-induced depression of glutamate was not a specific antagonism of glutamate. Dopamine, 60 nA (DA). Time in all traces 2 minutes.

Table 1 Neuronal responses to dopamine, amantadine and amphetamine

Drug	*Area*	*No. of cells*	*Responses No. (%)* +	*Responses No. (%)* —	*Responses No. (%)* *0*	*Depression of firing rate (spikes/s)**
Dopamine	Cortex	142	7 (5)	128 (90)	7 (5)	8 4±0.64
	Caudate	32	2 (6)	23 (72)	7 (22)	9.0±0.82
Amantadine	Cortex	129	5 (4)	108 (84)	16 (12)	15.2±1.08
	Caudate	32	1 (3)	26 (81)	5 (16)	13 0±1.05
Amphetamine	Cortex	83	7 (8)	70 (85)	6 (7)	14.8±1.15
	Caudate	12	0	12 (100)	0	15.5±1.46

* Mean ± s.e. of mean.
Responses are shown as: + = excitation; — = depression, *O* = no effect.

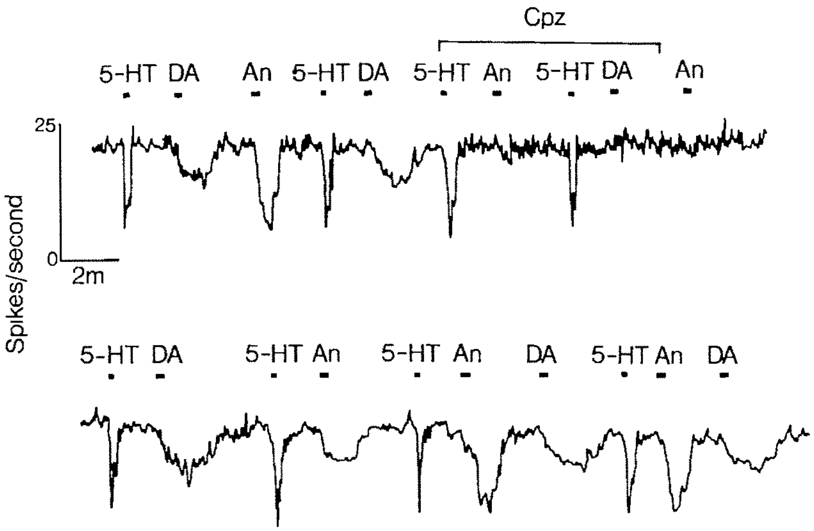


Figure 2 A recording of the firing rate of a cortical neurone, showing responses to 5-hydroxytryptamine, 60 nA (5-HT), dopamine, 60 nA (DA) and amantadine, 60 nA (An). The iontophoresis of chlorpromazine, 30 nA (Cpz), produces a reversible antagonism of dopamine and amantadine responses. The two parts of the record are consecutive. Time 2 minutes.

rate could be increased by increasing the ejection of glutamate or acetylcholine. This fact, and the absence of any change of spike height as monitored on the oscilloscopes, indicated that depression was not due to movement of the electrode, local anaesthetic effects on the cell membrane, or over-depolarization.

The latencies of dopamine responses were in the range 6–20 s, whereas latencies for amphetamine and amantadine responses were in the range 1–12 seconds.

Interactions of amantadine and dopamine

On a number of cells in both cerebral cortex and caudate nucleus regularly spaced, reproducible responses to dopamine were obtained and an ejection of amantadine was then made to determine whether the dopamine response could be changed by the presence of amantadine. In no case has a potentiation of dopamine resulted from this sequence of applications.

Dopamine receptor blocking agents

Chlorpromazine was applied to a total of 23 units (15 cortical) with currents of 30 nA. Larger current applications often led to direct neuronal depression and reduction of spike height, presumably as a result of chlorpromazine's local anaesthetic properties. Chlorpromazine blocked the depressant action of dopamine on 17 of the 23 cells (74%) and blocked amantadine depressions on 8 of 11 cells (72%) tested. No reduction of responses to 5-hydroxytryptamine were seen on any of 23 cells. Part of the record of the firing rate of a unit showing blockade of dopamine and amantadine whilst 5-hydroxytryptamine responses were unaffected, is shown in Figure 2.

Table 2 Concentrations of noradrenaline and dopamine in rat cerebral cortex

Pretreatment (i.p., 24 h previously)	*Noradrenaline (ng/g tissue)*	*Dopamine (ng/g tissue)*
Tween 80 1 ml/kg	283.1 ±30.4* (*n*=8)	183.0±25.5 (8)
Reserpine 10 mg/kg in tween 80	22.64± 4.8 (7)	14.62± 2.8 (7)
Saline 1 ml/kg	261.6±42.2 (6)	168 1±26.0 (6)
AMPT 200 mg/kg in saline	129 8±20.8 (4)	69.5± 8.7 (4)

* mean ± s.e. of mean.

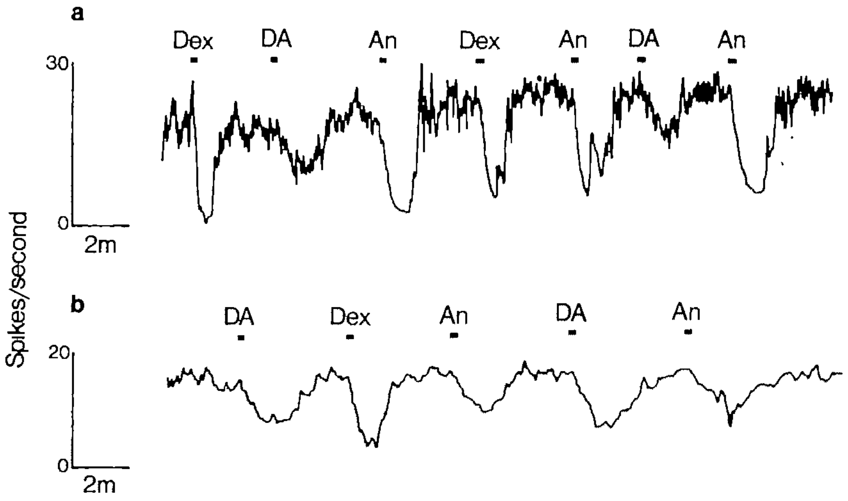


Figure 3 Records of the firing rates of two pyramidal tract neurones: (a) in a control rat pretreated with Tween 80 1 ml/kg 24 h before the experiment, (b) in a rat pretreated with reserpine 10 mg/kg in Tween 80, 24 h before the experiment. Responses to the three drugs are present after reserpine-treatment. The bars indicate the iontophoresis of amphetamine, 60 nA (Dex); dopamine, 60 nA (DA) and amantadine, 60 nA (An) Time 2 minutes.

Comparison of control and amine-depleted animals

Catecholamine concentrations. The results of the fluorimetric estimation of catecholamines in the cerebral cortex are shown in Table 2. After reserpine pretreatment noradrenaline and dopamine concentrations were both reduced by 92% compared with the Tween 80 controls. After AMPT injections, the amine levels were reduced by 50% and 59% respectively, compared with the saline controls.

Unit responses to iontophoresis. In this part of the experimental series the cells studied were cortical pyramidal tract cells. All iontophoretic ejections were

Table 3 Responses of pyramidal tract neurones to dopamine,amantadine and amphetamine in control and amine-depleted rats

*Pretreatment**	*Iontophoretic drug*	*No. of cells*	*Responses No. (%)* +	−	*0*
Tween 80	Dopamine	52	3 (6)	44 (85)	5 (9)
	Amantadine	50	1 (2)	40 (80)	9 (18)
	Amphetamine	44	0	40 (91)	4 (9)
Reserpine	Dopamine	40	1 (2)	33 (83)	6 (15)
	Amantadine	46	3 (6)	39 (85)	4 (9)
	Amphetamine	46	1 (2)	38 (83)	7 (15)
Saline	Dopamine	48	3 (6)	37 (77)	8 (17)
	Amantadine	36	0	29 (81)	7 (19)
	Amphetamine	30	0	27 (90)	3 (10)
α-methyl-*p*-tyrosine	Dopamine	30	3 (10)	24 (80)	3 (10)
	Amantadine	30	1 (3)	24 (80)	5 (17)
	Amphetamine	22	0	20 (91)	2 (9)

* Doses as in Table 2.
+ = excitation; − = depression; *0* = no effect.

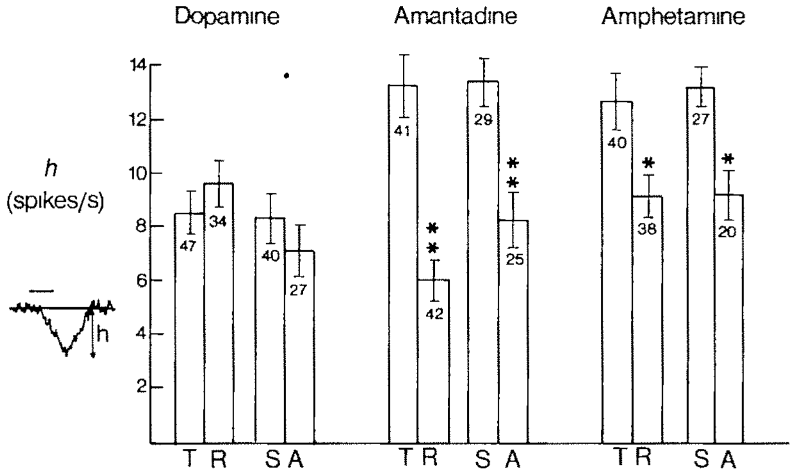


Figure 4 Histograms showing the values obtained for *h*, the maximum change of firing rate produced, resulting from the iontophoresis of dopamine, amantadine and amphetamine. The height of each column indicates the mean value, the vertical bar shows ± s.e. of the mean, and the number within each column indicates the number of cells used in the calculations. T=Tween 80 pretreatment; R=reserpine pretreatment; S=saline pretreatment; A=α-methyl-p-tyrosine pretreatment.
* $P<0.02$; ** $P<0.001$

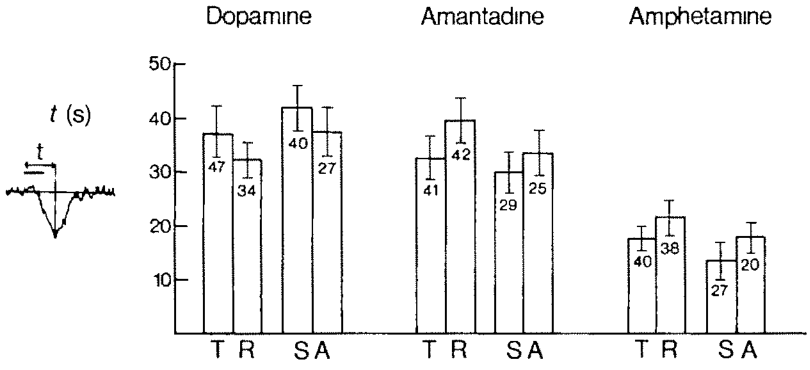


Figure 5 Histograms showing the values obtained for *t*, the time between beginning a drug ejection and the peak change of firing produced Details as for Figure 4

effected with currents of 60 nA for 15 seconds. The numbers of units tested and their responses to the various agonists are presented in Table 3. The percentage figures are similar to those for the normal untreated animals discussed above (Table 1).

Figure 3 shows records of the firing rates of two units, one in a control animal and one in a reserpine-treated animal, to illustrate the absence of any marked difference in the responses.

The values obtained for the parameters *h*, *t* and *d* are most conveniently summarized in histogram form in Figures 4, 5 and 6 respectively. There was a significant reduction in the maximum responses (*h*) to amantadine ($P<0.001$) and amphetamine ($P<0.02$) in the amine-depleted compared with control rats (Figure 4). There was no statistically significant change in the parameters *t* (Figure 5) and *d* (Figure 6) for amphetamine and amantadine, and no significant change was apparent for any of the three parameters in the case of dopamine.

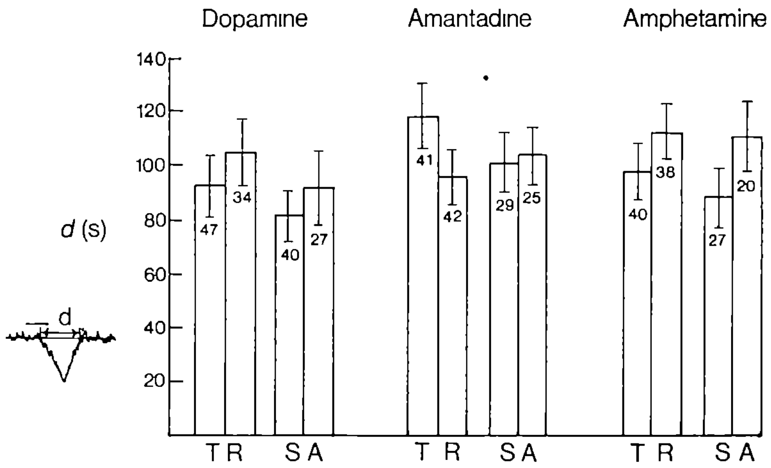


Figure 6 Histograms showing the values obtained for *d*, the total duration of drug response. Details as for Figure 4.

Discussion

Most of the available evidence on the mechanism of action of amantadine indicates an action primarily on the dopaminergic systems of the brain (Strömberg & Svensson, 1971; Von Voigtlander & Moore, 1973). The present results support this idea by demonstrating that iontophoretically applied amantadine had the same effect as dopamine and amphetamine on neurones in the cerebral cortex and caudate nucleus. The usual effect of these substances was depression of neuronal firing rate. Since amphetamine and amantadine can act on noradrenergic as well as dopaminergic neurones (Glowinski, 1970; Sinclair, 1973; Papeschi, 1974) this similarity of action is particularly significant in the caudate nucleus where dopamine is the predominant catecholamine, and only minute quantities of noradrenaline are present (Vogt, 1954; Bertler & Rosengren, 1959).

The similarity of action is more difficult to interpret in the cerebral cortex since there are both noradrenaline-containing and dopamine-containing nerve terminals present (Fuxe, Hamberger & Hökfelt, 1968; Lindvall, Björklund, Moore & Stenevi, 1974). Nevertheless the similarity of action of dopamine and amantadine can be considered meaningful in the light of the additional observation that responses to both substances could be blocked by chlorpromazine at a time when responses to 5-hydroxytryptamine were unaltered. Chlorpromazine has been shown previously to block dopamine depressions of unit firing in the striatum (York, 1972) and cerebral cortex (Stone, 1974), but has little effect on noradrenaline depressions in the cortex (Stone, 1973a).

Although cholinergic nerve endings are present in the cortex and striatum, amantadine's effects are not likely to be due to an effect on such terminals, since amantadine has been shown to have very little effect on peripheral cholinergic systems (Vernier, Harmon, tump, Lynes, Marvel & Smith, 1969; Grelak *et al.*, 1970; Bianchi & Tomasi, 1973).

The primarily depressant action of dopamine on striatal neurones is consistent with the idea that most nigrostriatal dopaminergic neurones are inhibitory to caudate cells (McLennan & York, 1967; Connor, 1970). The results are also in agreement with previous studies of the effects of iontophoretically applied dopamine. McLennan & York (1967), for example, found that most caudate units (60%) were depressed by dopamine and only 9% were excited. Connor (1970) found that 68% of caudate units were depressed by dopamine. Many cortical units have also been shown to be depressed by dopamine (Krnjević & Phillis, 1963).

The significance of the excitatory responses to the three agonists is not clear. They were observed too rarely for attempts at antagonism or the assessment of changes after amine depletion to be meaningful. It is not likely that the excitatory responses indicate an excitatory dopaminergic nigrostriatal pathway. The possible existence of such a pathway was mentioned by Feltz (1971) following his demonstration of a monosynaptic excitatory nigrostriatal pathway which could be blocked by haloperidol. Feltz & de Champlain (1972a) later showed that the transmitter mediating this excitation was probably not a catecholamine since the excitation persisted after the degeneration of catecholamine containing neurones induced by 6-hydroxydopamine.

The most likely explanation of the excitations seen in the present experiments is that they are the result of an indirect action of the drugs causing depression of a nearby neurone which has an inhibitory influence on the unit being recorded.

It is generally accepted that neuronal uptake processes are responsible for the removal of transmitter catecholamines from the synaptic cleft (Iversen, 1967). Hence drugs which inhibit the uptake process can usually be shown to potentiate the actions of the catecholamines (Bradshaw, Roberts & Szabadi, 1971). In the present experiments, amantadine, in doses sufficient to cause some neuronal depression, did not produce any apparent potentiation of dopamine responses. This suggests that amantadine has very little, if any, ability to inhibit the neuronal uptake of dopamine. This conclusion has been reached by most other groups in direct biochemical experiments (Fletcher & Redfern, 1970; Strömberg & Svensson, 1971; Baldessarini *et al.*, 1972; Heikkila & Cohen, 1972).

Studies of amine-depleted rats

As a comparison of Tables 1 and 3 shows, the proportions of cortical cells responding to dopamine, amantadine and amphetamine were similar in the various groups of pretreated rats (Table 3) and in the original sample of normal untreated animals (Table 1). This may be considered to indicate that none of the pretreatment injections caused a gross disturbance in the pattern of neuronal sensitivity.

Dopamine responses appeared to be unchanged by amine depletion. Certainly no reduction in this response would be expected since dopamine is assumed to act postsynaptically and its effects should therefore be independent of presynaptic amine stores. Ungerstedt (1971) has shown that following a single intraperitoneal injection of reserpine 10 mg/kg, there is a marked supersensitivity of dopamine receptors after 24 hours. Although in the present experiments there is a slight increase in the value of h for dopamine in reserpine-treated animals compared with controls, the increase is not statistically significant. However, the supersensitivity described by Ungerstedt (1971) was seen as turning behaviour of unilaterally striatotomized rats after the injection of normally subthreshold doses of apomorphine (0.25 mg/kg). The supersensitivity therefore was of striatal dopamine receptors. It is possible that receptors in the cerebral cortex developed supersensitivity with a slower time course.

The significant reduction of neuronal responses to amantadine and amphetamine probably indicates a substantial contribution of an indirect amine-releasing effect to their mechanism of action. Such a conclusion would be consistent with previous studies of the action of both amantadine (Grelak *et al.*, 1970; Strömberg, Svensson & Waldeck, 1970; Farnebo *et al.*, 1971) and amphetamine (Carlsson *et al.*, 1965, 1966; Fuxe & Ungerstedt, 1968; Boakes *et al.*, 1972).

The residual effects of these substances after amine depletion suggest that they may also exert a direct action on postsynaptic receptors. A direct action of amantadine has been postulated by several workers who found that the drug still had anticataleptic properties after central catecholamine depletion by AMPT or reserpine (Cashin & Sutton, 1973; Maj, Sowińska & Baran, 1973; Papeschi, 1974). Similarly, from an examination of the locomotor activity of mice or rats it has been concluded that amphetamine can directly stimulate catecholamine receptors (Smith, 1963; Van Rossum & Hurkmans, 1964).

An alternative explanation of the residual responses to amantadine and amphetamine is that the small amount of catecholamine remaining after reserpine is sufficient to maintain a store of amine in a form which can be released by amantadine and amphetamine.

However, several other groups have found no difference between neuronal responses to amphetamine in control rats and rats pretreated with reserpine or 6-hydroxydopamine (Hoffer, Siggins & Bloom, 1971; Feltz & de Champlain, 1972b; Kostopoulos & Yarbrough, 1974). 6-Hydroxydopamine produces a profound loss of catecholamines and aminergic terminals from treated areas of the brain, so that these observations support the possibility that amphetamine can act directly on postsynaptic receptors. The results obtained in the present study that amantadine can produce neuronal responses after reserpine-treatment, which are comparable to those produced by amphetamine provides suggestive evidence that amantadine can also act in this way.

I am grateful to Geigy Labs Ltd. for the gift of amantadine hydrochloride. I should like to thank Professor P.B. Bradley and Dr N. Davidson for their helpful criticisms of the manuscript.

References

ANDÉN, N-E., DAHLSTRÖM, A., FUXE, K. & LARSSON, K. (1966). Functional role of the nigrostriatal dopamine neurones. *Acta pharmac. tox.*, **24**, 263–274.

ARBUTHNOTT, G.W. & CROW, T.J. (1971). The relationship between turning behaviour and unilateral release of dopamine in the rat. *Exp. neurol.*, **30**, 484–491.

BALDESSARINI, R.J., LIPINSKI, J.F & CHACE, K.V. (1972). Effects of amantadine hydrochloride on catecholamine metabolism in the brain of the rat. *Biochem. Pharmac.*, **21**, 77–87.

BERTLER, A. & ROSENGREN, E. (1959). Occurrence and distribution of catecholamines in brain. *Acta physiol. scand.*, **47**, 350–361.

BIANCHI, C. & TOMASI, L. (1973). Central nervous system and autonomic nervous system effects of amantadine and of some standard anti-Parkinson drugs. *Pharmacology*, **10**, 226–237.

BIRDWOOD, G.F.B., GILDER, G.S.S. & WINK, C.A S. (1971). *Parkinson's disease—a new approach to treatment.* London: Academic Press.

BOAKES, R.J., BRADLEY, P.B. & CANDY, J.M. (1972). A neuronal basis for the alerting action of (+)-amphetamine. *Br. J. Pharmac.*, **45**, 391–403.

BRADSHAW, C.M., ROBERTS, M.H.T. & SZABADI, E. (1971). Effect of tricyclic antidepressants on monoamine responses of single cortical neurones. *Br. J. Pharmac.*, **41**, 394–395P.

CALNE, D.B. (1970). *Parkinsonism: Physiologh, gvvpharmacology and treatment.* London: Arnold.

CARLSSON, A., LINDQVIST, M., DAHLSTRÖM, A., FUXE, K & MASUOKA, D. (1965). Effect of the amphetamine group on intraneuronal brain amines *in vivo* and *in vitro*. *J. Pharm. Pharmac.*, **17**, 521–523.

CARLSSON, A , LINDQVIST, M., FUXE, K. & HAMBERGER, B. (1966). The effect of (+)-amphetamine on various central and peripheral catecholamine-containing neurones. *J. Pharm. Pharmac.*, **18**, 128–130.

CASHIN, C.H. & SUTTON, S. (1973). The effect of anti-Parkinson drugs on catalepsy induced by α-methyl-*p*-tyrosine in rats pretreated with intraventricular 6-hydroxydopamine. *Br. J. Pharmac.*, **47**, 658–659P.

CHRISTIE, J.E. & CROW, T.J. (1971). Turning behaviour as an index of the action of amphetamines and ephedrines on central dopamine-containing neurones. *Br. J. Pharmac.*, **43**, 658–667.

CONNOR, J.D. (1970). Caudate nucleus neurones: correlation of the effects of substantia nigra stimulation with iontophoretic dopamine. *J. Physiol.*, **208**, 691–703.

COX, R.H. & PERHACH, J.L. (1973). A sensitive, rapid and simple method for the simultaneous spectrophotofluorimetric determinations of norepinephrine, dopamine, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in discrete areas of brain. *J. Neurochem.*, **20**, 1777–1780.

COX, B. & THA, S.J. (1973). Effects of amantadine and 1-dopa on apomorphine and d-amphetamine induced stereotyped behaviour in rats. *Eur. J. Pharmac.*, **24**, 96–100.

CROW, T.J. (1971). The relationship between lesion site, dopamine neurones and turning behaviour in the rat. *Exp. neurol.*, **32**, 247–255.

FARNEBO, L., FUXE, K., GOLDSTEIN, M., HAMBERGER, B. & UNGERSTEDT, U. (1971). Dopamine and noradrenaline releasing action of amantadine in the central and peripheral nervous system: a possible mode of action in Parkinson's disease. *Eur. J. Pharmac.*, **16**, 27–38.

FELTZ, P (1971). Sensitivity to haloperidol of caudate neurones excited by nigral stimulation. *Eur. J. Pharmac.*, **14**, 360–364.

FELTZ, P. & DE CHAMPLAIN, J. (1972a). Persistence of caudate unitary responses to nigral stimulation after destruction and functional impairment of the striatal dopaminergic terminals. *Brain Res.*, **43**, 595–600.

FELTZ, P. & DE CHAMPLAIN, J. (1972b). Enhanced sensitivity of caudate neurones to microiontophoretic injections of dopamine in 6-hydroxydopamine treated rats. *Brain Res.*, **43**, 601–605.

FIFKOVÁ, E. & MARSALA, J. (1967). Stereotaxic atlas of the rat. In *Electrophysiological methods in biological research*, ed. Bureš, J., Petráň, M. & Zachar, J., pp. 653–695. Prague: Academic Press.

FLETCHER, E.A. & REDFERN, P.H. (1970). The effects of amantadine on the uptake of dopamine and noradrenaline by rat brain homogenates. *J. Pharm. Pharmac.*, **22**, 957–959.

FUXE, K., HAMBERGER, B. & HÖKFELT, T. (1968). Distribution of noradrenaline nerve terminals in cortical areas of the rat. *Brain Res.*, **8**, 125–129.

FUXE, K. & UNGERSTEDT, U. (1968). Histochemical studies on the effects of (+)-amphetamine, drugs of the imipramine group and tryptamine on central catecholamine and 5-hydroxytryptamine neurones after intraventricular injection of catecholamines and 5-hydroxytryptamine. *Eur. J. Pharmac.*, **4**, 135–144.

GLOWINSKI, J. (1970). Effects of amphetamine on various aspects of catecholamine metabolism in the central nervous system of the rat. In *Amphetamines and related compounds*, ed. Costa, E. & Garattini, S., pp. 301–316. New York: Raven Press.

GRELAK, R.P., CLARK, R., STUMP, J.M. & VERNIER, V.G. (1970). Amantadine-dopamine interaction: Possible mode of action in Parkinsonism. *Science*, **169**, 203–204.

HEIKKILA, R.E. & COHEN, G. (1972). Evaluation of amantadine as a releasing agent or uptake blocker for ^{3}H-dopamine in rat brain slices. *Eur. J. Pharmac.*, **20**, 156–160.

HEIMANS, R.L.H., RAND, M.J. & FENNESSY, M.R. (1972). Effects of amantadine on uptake and release of dopamine by a particulate fraction of rat basal ganglia. *J. Pharm. Pharmac.*, **24**, 875–879.

HOFFER, B.J., SIGGINS, G.R. & BLOOM, F.E. (1971). Studies on norepinephrine-containing afferents to Purkinje cells of rat cerebellum. II. Sensitivity of Purkinje cells to norepinephrine and related substances administered by microiontophoresis. *Brain Res.*, **25**, 522–534.

HORNYKIEWICZ, O. (1966). Dopamine (3-hydroxytyramine) and brain function. *Pharmac. Rev.*, **18**, 925–964.

IVERSEN, L.L. (1967). *The uptake and storage of noradrenaline in sympathetic nerves.* London: Cambridge Univ. Press.

KOSTOPOULOS, G.K. & YARBROUGH, G.G. (1974). Iontrophoretic studies with 'false transmitters' on cerebellar Purkinje cells. *Br. J. Pharmac.*, **52**, 136P.

KRNJEVIĆ, K. & MITCHELL, J.F. (1961). A simple and reliable device utilizing transistors for the maintenance of a constant body temperature. *J. Physiol.*, **158**, 6–8P.

KRNJEVIĆ, K. & PHILLIS, J.W. (1963). Actions of certain amines on cerebral cortical neurones. *Br. J. Pharmac. Chemother.*, **20**, 471–490.

LASSEN, J.B. (1973). The effect of amantadine and (+)-amphetamine on motility in rats after inhibition of monoamine synthesis and storage. *Psychopharmacologia*, **29**, 55–64.

LINDVALL, O., BJÖRKLUND, A., MOORE, R.Y. & STENEVI, U. (1974). Mesencephalic dopamine neurons projecting to neocortex. *Brain Res.*, **81**, 325–331.

McLENNAN, H. & YORK, D.H. (1967). The action of dopamine on neurones of the caudate nucleus. *J. Physiol.*, **189**, 393–402.

MAJ, J, SOWIŃSKA, H. & BARAN, H. (1973). Effects of amantadine, amphetamine and apomorphine on the locomotor activity in rats. *Life Sci.*, **12**, 511–518.

MOORE, K.E. (1963). Toxicity and catecholamine releasing action of d- and l-amphetamine in isolated and aggregated mice. *J. Pharmac. exp. Ther.*, **142**, 6–12.

PAPESCHI, R. (1974). Amantadine may stimulate dopamine and noradrenaline receptors. *Neuropharmacology*, **13**, 77–84.

SCATTON, B., CHERAMY, A, BESSON, M.J. & GLOWINSKI, J. (1970).Increased synthesis and release of dopamine in the striatum of the rat after amantadine treatment. *Eur. J. Pharmac.*, **13**, 131–133.

SCHWAB, R.S., ENGLAND, A.C., POSKANZER, D.C. & YOUNG, R.R (1969). Amantadine in the treatment of Parkinson's disease. *J. Am. Med. Ass.*, **208**, 1168–1170.

SINCLAIR, J.G. (1973). Interaction of amantadine with tyramine and noradrenaline on the cat nictitating membrane. *Canad. J. Physiol. pharmac.*, **51**, 305–307.

SMITH, C B. (1963). Enhancement by reserpine and α-methyldopa of the effect of d-amphetamine upon the locomotor activity of mice. *J. Pharmac. exp. Ther.*, **142**, 343–350.

SPILKER, B.A. & DHASMANA, K.M. (1974). On the specificity of dopamine release by amantadine. *Experientia*, **30**, 64–65.

STONE, T W. (1972a). Cholinergic mechanisms in the rat somato-sensory cerebral cortex. *J. Physiol.*, **225**, 485–499.

STONE, T.W. (1972b). Cortical responses to pyramidal tract stimulation in the rat. *Exp. neurol.*, **35**, 492–502.

STONE, T.W. (1973a). Pharmacology of pyramidal tract cells in the cerebral cortex. Noradrenaline and related substances. *Naunyn-Schmiedeberg's Arch. Pharmac.*, **278**, 333–346.

STONE, T.W. (1973b). Cortical pyramidal tract interneurones and their sensitivity to L-glutamic acid. *J. Physiol.*, **233**, 211–225.

STONE, T.W (1974). Further evidence for a dopamine receptor stimulating action of an ergot alkaloid. *Brain Res.*, **77**, 177–180.

STONE, T.W. (1975). Responses of cortical pyramidal tract cells to amantadine and amphetamine after depletion of central catecholamines. *Br. J. Pharmac.*, **54**, 244–245P.

STRÖMBERG, U. & SVENSSON, T.H. (1971). Further studies on the mode of action of amantadine. *Acta pharmac. tox.*, **30**, 161–171.

STRÖMBERG, U., SVENSSON, T.H. & WALDECK, B (1970). On the mode of action of amantadine. *J. Pharm. pharmac.*, **22**, 959–962.

UNGERSTEDT, U (1971). Postsynaptic supersensitivity after 6-hydroxydopamine induced degeneration of the nigrostriatal dopamine system. *Acta physiol. scand.*, **82**, Suppl. 367, 69–93.

VAN ROSSUM, J.M. (1966). The significance of dopamine receptor blockade for the mechanism of action of neuroleptic drugs. *Arch. int. pharmacodyn*, **160**, 492–494.

VAN ROSSUM, J.M & HURKMANS, J.A.TH.M. (1964). Mechanism of action of psychomotor stimulant drugs. *Int. J. neuropharmac.*, **3**, 227–239.

VERNIER, V.G., HARMON, J.B , STUMP, J.M., LYNES, T.E., MARVEL, J.P & SMITH, D.H. (1969). The toxicologic and pharmacologic properties of amantadine hydrochloride. *Toxicol. appl. pharmac.*, **15**, 642–665.

VOGT, M. (1954). The concentration of sympathin in different parts of the central nervous system under normal conditions and after the administration of drugs. *J. Physiol.*, **123**, 451–481.

VON VOIGTLANDER, P.F. & MOORE, K.E. (1973). Involvement of nigrostriatal neurons in the *in vivo* release of dopamine by amphetamine, amantadine and tyramine. *J. Pharmac. exp. Ther.*, **184**, 542–552.

YORK, D.H. (1972). Dopamine receptor blockade—a central action of chlorpromazine on striatal neurones. *Brain Res.*, **37**, 91–100.

(Received July 8, 1975)

Br. J. Pharmac. (1976), **56**, 111–113

EFFECT OF ATROPINE ON THE DECAY OF MINIATURE END-PLATE CURRENTS AT THE FROG NEUROMUSCULAR JUNCTION

ANNE FELTZ & W.A. LARGE
Laboratoire de Neurobiologie, Ecole Normale Supérieure, 46, Rue d'Ulm, 75005, Paris, France

1 The effect of atropine on the time constant of decay (τ) of miniature end-plate currents was investigated.

2 The atropine-induced shortening of τ was accompanied by a reduction in the voltage-sensitivity of τ.

3 This effect of atropine was reversible and dose-dependent, the voltage sensitivity being abolished with high concentrations of atropine (2.5×10^{-5} M).

Introduction

The time constant of decay (τ) of synaptic currents at the neuromuscular junction has been shown to be markedly affected by the post-synaptic membrane potential, being prolonged by a hyperpolarization of the membrane (Gage & Armstrong, 1968; Kordaš, 1969). Making certain assumptions about the fundamental processes underlying the end-plate current (e.p.c.), Magleby & Stevens (1972a, b) demonstrated that τ reflects the life-time of the acetylcholine-induced elementary current pulse; moreover, in 1973, Anderson & Stevens showed that the τ of e.p.cs, the τ of miniature end-plate currents (m.e.p.cs) and the channel lifetime, as deduced from noise analysis, were similarly affected by alteration of the membrane potential.

The action of atropine in reducing the amplitude of miniature and nerve-evoked end-plate potentials at the neuromuscular junction is characterized by a diminution of the decay times of the potentials (Beránek & Vyskočil, 1968). The shortening of the time-course of e.p.cs (Kordaš, 1968; Magazanik & Vyskočil, 1969) can be accounted for by a reduction in the lifetime of the elementary current pulse produced by acetylcholine (ACh), as revealed from noise analysis (Katz & Miledi, 1973). In the present paper, we describe the action of atropine on the voltage-sensitivity of $\tau_{\text{m.e.p.cs}}$

Methods

M.e.p.cs were studied in the cutaneous pectoris muscle of the frog, *Rana esculenta*, by two techniques: (1) focal extracellular recording with glass microelectrodes filled with 2 M NaCl (tip diameter 5–10 µm); (2) intracellularly, by the use of the two electrodes voltage-clamp method. In the latter case the two electrodes (filled with 3 M KCl) were inserted into the end-plate of the muscle fibre within 50–100 µm of each other. A rise time of around 0.5 ms for the m.e.p.cs was used as an index of close vicinity of the releasing sites. The preparation was constantly superfused and all experiments were carried out at room temperature (20–23 °C). The composition of the Ringer was (mM): NaCl 161, $CaCl_2$ 1.8, KCl 2.5, Tris maleate 2, acetylglycine 2. The pH was adjusted to 7.2 by addition of NaOH. The extra NaCl was added to increase the osmotic pressure of the bathing fluid and hence the frequency of miniature end-plate potentials. Atropine sulphate (Serlabo) was diluted in the bathing fluid at the given concentrations. The m.e.p.cs were photographed directly on to moving film and subsequently were enlarged and traced on to graph paper. The decays of the currents were plotted according to the equation: $I_t = I_0 e^{-t/\tau}$ on a 9810A Hewlett Packard desk computer. I_t = current at time t; I_0 = current at time zero (i.e. the peak current) and τ is time constant of decay. For each experiment, the decay constants of at least 6 m.e.p.cs were calculated and averaged for each parameter. Unless stated, the mean ±s.e. of decay time constant was calculated from at least 6 cells for each experimental condition.

Results

In normal Ringer solution, at the resting membrane potential (~−90 mV), the time constant of decay

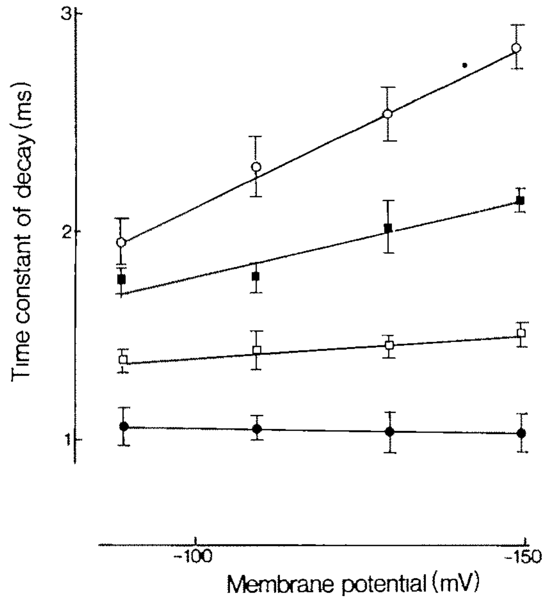


Figure 1 Effect of atropine sulphate on the relation between the time constant of decay of miniature end-plate currents and clamp potential. Control experiments (○); measurements in presence of atropine sulphate: (■) 5×10^{-6} M; (□) 1×10^{-5} M; (●) 2.5×10^{-5} M. Vertical bars represent ±s.e. mean.

of m.e.p.cs in voltage-clamp experiments was 2.20 ± 0.10 ms (mean ± s.e. from 26 cells). This was in good agreement with the value obtained with focal external recording 2.21 ± 0.08 (18 cells). This would suggest that in the voltage-clamp experiments, the electrodes were placed in the end-plate region and also that the membrane potential was adequately controlled (see Magleby & Stevens, 1972a). When atropine was added to the bathing fluid the amplitude of the m.e.p.cs was reduced and there was a concomitant shortening of the time course of decay. These effects were dose-dependent with a threshold concentration of about 5×10^{-6} M. The effects of atropine developed within 2–3 min and appeared to be maximal after 10 min when all measurements were made. In voltage-clamped fibres, the time constants of decay after 1×10^{-5} and 2.5×10^{-5} M were 1.39 ± 0.05 and 1.09 ± 0.08 ms respectively (compared with 2.20 of controls, see above) and in the extracellular experiments the same concentrations of atropine produced τ values of 1.32 ± 0.08 and 1.20 ± 0.05 ms respectively (control = 2.21). However, it is important to note that even at high concentrations of atropine the falling phase of currents conformed to a single exponential and hence atropine differs in its action from the local anaesthetics (see review by Rang, 1975).

It has been shown that in normal conditions $\tau_{\text{m.e.p.cs}}$ is sensitive to changes in the membrane potential (Anderson & Stevens, 1973; Gage & McBurney, 1975). Furthermore, over a wide range of membrane potentials, τ can be related to the membrane potential by the equation $\tau_V = \tau_0 e^{V/H}$ where τ_V and τ_0 are the time constants of decay at potentials V and 0 respectively and H is a constant which indicates the voltage-sensitivity of the falling phase of m.e.p.cs (notation of Gage & McBurney, 1975, after Magleby & Stevens, 1972a,b). We have applied the same analysis in the present experiments. Figure 1 shows the change in the time constant of decay, when the membrane potential is hyperpolarized from the holding potential of −90 mV in 20 mV steps before and after various concentrations of atropine. In 17 cells, H had a mean value of -180 ± 13 mV and the mean τ_0 was 1.26 ± 0.09 ms (for each cell, τ_0 was calculated by extrapolation using the least squares method). In the presence of atropine there was a concentration-dependent reduction in the voltage-sensitivity and with 2.5×10^{-5} M atropine sulphate, voltage-sensitivity was abolished. It is interesting to note that assuming the exponential relationship between τ and membrane potential, atropine did not alter the extrapolated τ_0 value (the τ_0 values for the 5×10^{-6}; 1×10^{-5} and 2.5×10^{-5} M atropine were respectively 1.25 ± 0.05; 1.31 ± 0.07 and 1.17 ± 0.09 ms. Both the shortening and the reduction in voltage-sensitivity of τ produced by atropine were reversible and the control values were attained after 10–20 min washing.

Discussion

The results show that the atropine-induced shortening of the time course of miniature end-plate currents is accompanied by a reduction in the voltage-sensitivity of the decay constant. Moreover the action of atropine appears to be different from that of local anaesthetics (Maeno, 1966) inasmuch as the decay remains a simple exponential after the treatment with atropine whereas the latter drugs cause a biphasic decay. In this respect atropine resembles octanol which shortens miniature currents (Gage, McBurney & Van Helden, 1974) and also reduces voltage-sensitivity (Feltz & Large, unpublished results). The loss of voltage-sensitivity after atropine may be explained by a major decrease in the lifetime of the end-plate ionic channels such that another process, for example diffusion of ACh out of the cleft, becomes the rate limiting factor. An alternative explanation may be a direct action of atropine on the ACh-receptor complex and/or the ionic channel. It is interesting to note that at some other synapses, there is no voltage-sensitivity of the time course of the decay phase (Gardner & Stevens, 1974; Llinás, Joyner & Nicholson, 1974), and from our results with atropine it would appear that ACh can open the channels at the neuromuscular junction also when voltage-sensitivity has been abolished. Therefore voltage-sensitivity does not appear to be a fundamental characteristic of synapses.

W.A.L was a MRC Travelling Fellow. This work was supported by grants of INSERM (N° 75-4-200-6) and of CNRS (ERA 329 and ATP Médiateurs N° 18-58).

References

ANDERSON, C.R. & STEVENS, C.F. (1973). Voltage clamp analysis of acetylcholine-produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol.*, **235**, 655–691.

BERÁNEK, R. & VYSKOČIL, F. (1968). The effect of atropine on the frog sartorius neuromuscular junction. *J. Physiol.*, **195**, 493–503.

GAGE, P.W. & ARMSTRONG, C.M. (1968). Miniature end-plate currents in voltage-clamped muscle fibres. *Nature, Lond.*, **218**, 363–365.

GAGE, P.W. & McBURNEY, R.N. (1975). Effects of membrane potential, temperature and neostigmine on the conductance change caused by a quantum of acetylcholine at the toad neuromuscular junction. *J. Physiol.*, **244**, 385–408.

GAGE, P.W., McBURNEY, R.N. & VAN HELDEN, D. (1974). End-plate currents are shortened by octanol: possible role of membrane lipid. *Life Sciences*, **14**, 2277–2283.

GARDNER, D. & STEVENS, C.F. (1974). Analysis of a cholinergic inhibitory postsynaptic conductance. *Fed. Proc.*, **33**, 450.

KATZ, B. & MILEDI, R. (1973). The characteristics of 'end-plate noise' produced by different depolarizing drugs. *J. Physiol.*, **230**, 707–717.

KORDAŠ, M. (1968). The effect of atropine and curarine on the time course of the end-plate potential in frog sartorius muscle. *Int. J. Neuropharmac.*, **7**, 523–530.

KORDAŠ, M. (1969). The effect of membrane polarization on the time course of the end-plate current in frog sartorius muscle. *J. Physiol.*, **204**, 493–502.

LLINÁS, R., JOYNER, R.W. & NICHOLSON, C. (1974). Equilibrium potential for the synaptic response in the squid giant synapse. *J. gen. Physiol.*, **64**, 519–535.

MAENO, T. (1966). Analysis of sodium and potassium conductances in the procaine end-plate potential. *J. Physiol.*, **183**, 592–606.

MAGAZANIK, L.G. & VYSKOČIL, F. (1969). Different actions of atropine and some analogues on the end-plate potentials and induced acetylcholine potentials. *Experientia*, **25**, 618–619.

MAGLEBY, K.L. & STEVENS, C.F. (1972a). The effect of voltage on the time course of end-plate currents. *J. Physiol.*, **223**, 151–171.

MAGLEBY, K.L. & STEVENS, C.F. (1972b). A quantitative description of end-plate currents. *J. Physiol.*, **223**, 173–197.

RANG, H.P. (1975). Acetylcholine receptors. *Quart. Rev. Biophys.*, **7**, 283–399.

(Received July 24, 1975.
Revised August 29, 1975)

Br. J. Pharmac. (1976), **56**, 115–123

THE INFLUENCE OF MOTOR NERVE TETANIZATION ON THE EFFECT OF ORGANOPHOSPHORUS CHOLINESTERASE INHIBITORS ON NEUROMUSCULAR TRANSMISSION

A.F. DANILOV, YU.J. IVANOV & M.J. MICHELSON
Pharmacological Laboratory, Sechenov Institute of Evolutionary Physiology and Biochemistry, Leningrad, K–223, Academy of Sciences, USSR

1 The changes in the activity of synaptic cholinesterases (ChE) of the ant. tibial muscle in the anaesthetized cat were detected by recording the changes of the blocking activity of acetylcholine (ACh) and comparing them with the blocking activity of carbaminoylcholine (carbachol) injected intra-arterially or intravenously. After the administration of organophosphorus inhibitors (OPI) of ChE the ACh blocking dose diminished 500 to 2000-fold but the carbachol blocking dose did not change. In 4–6 h after the injection of OPI the ACh blocking dose increased again 8 to 15-fold, but the dose of carbachol still remained unchanged. The transmission of high frequency impulses improved after OPI in parallel with the decrease of the ACh blocking activity. Thus the synaptic ChE is partly restored in a few hours after its irreversible inhibition with OPI.

2 Tetanization of the motor nerve (50–60 Hz, 10 min), started simultaneously with the intravenous injection of OPI (armine, Gd–42), diminished the impairment of neuromuscular transmission. On the side of tetanization the ACh blocking action was less pronounced and the transmission of high frequency impulses better than on the control side. Thus the tetanization produced some protection of synaptic ChE against inhibition by OPI. The protective effect of tetanization was absent when the tetanization was performed before the injection of OPI or was started 10–20 min after the injection of OPI.

3 The protective effect of tetanization was also observed on the isolated phrenic nerve diaphragm preparation of the rat.

4 The possible mechanisms of the protective effect of tetanization are discussed.

Introduction

The irreversible organophosphorus inhibitors (OPI) of cholinesterases (ChE) can produce a reversible block of neuromuscular transmission. The reversibility of the block produced by OPI has been shown by many authors, but in most cases the restoration of neuromuscular transmission was not accompanied by a detectable rise in muscle ChE activity (Berry & Evans, 1951; McNamara, Murtha, Bergner, Robinson, Bender & Wills, 1954; Meeter & Wolthuis, 1968; Meeter, 1969). However, it is very difficult to evaluate quantitatively the activity of synaptic ChE in the muscle. The histochemical method is not quantitative. With homogenates of the whole muscle it is possible to determine biochemically only the total cholinesterase activity, synaptic and extrasynaptic, including the intracellular enzyme which does not play any functional role (Koelle, 1963). Barstad (1960) showed that only a slight increase of ChE activity was necessary to restore the neuromuscular transmission impaired by DFP. Such a minute degree of ChE reactivation may be undetectable with the usual biochemical methods.

Comparison of the neuromuscular blocking doses of acetylcholine (ACh) and carbaminoylcholine (carbachol) in the whole organism can give an idea of the activity of synaptic ChE. The drugs can be injected intra-arterially, that is, directly to the muscle (Danilov, 1967), or intravenously. With intravenous injection ACh is also hydrolysed in other tissue although the role of blood in the hydrolysis is not great (Michelson, Muske & Protas, 1974). Using our *in vivo* method (see methods section) we found that the synaptic ChE, having been almost totally inactivated by OPI, could be spontaneously restored to a certain degree during the first few hours after the administration of OPI.

It is well known that ACh can protect ChE from irreversible inactivation by OPI (see, for instance, Koelle, 1946; Augustinsson & Nachmansohn, 1949; Burgen, 1949; Aldridge, 1950; Brestkin, Volkova &

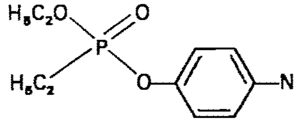
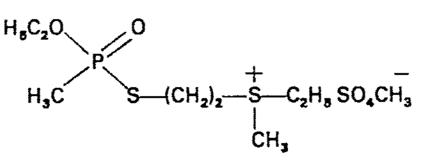
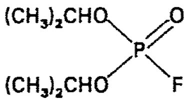
Rosengart, 1964, Volkova, 1965). The protective action of ACh was also shown on the isolated diaphragm of the rat (Barstad, 1960; Mittag, Ehrenpreis & Hehir, 1971) and on mouse diaphragm (Ostrowski & Barnard, 1961). It is possible that in the intact muscle the transmitter which accumulates in the synapse during the action of OPI can protect the synaptic ChE from irreversible inhibition by OPI. The accumulation of ACh in synapses during the action of OPI can be accelerated by tetanization of the motor nerve which increases the release of ACh from the nerve endings in a unit of time (Straughan, 1960, Krnjević & Mitchell, 1961; Bowman & Hemsworth, 1965). Thus, tetanization of the motor nerve should enhance the protective action of the synaptic ACh against the irreversible inactivation of synaptic ChE by OPI. This possibility was tested experimentally.

Elucidation of the protective properties of synaptic ACh may help to explain the reversibility of the transmission blockade produced by OPI. Some preliminary results of these experiments have been published (Ivanov, 1972; Danilov & Ivanov, 1972; Ivanov, Danilov, Zelixon & Michelson, 1972).

Methods

Cats (2 to 4 kg) of either sex were anaesthetized with urethane (500 mg/kp, i.p.) and chloralose (50 mg/kg, i.p.) and injected with atropine sulphate (7 mg/kg, i.m.). The trachea was cannulated and artificial respiration was given. The peroneal nerve(s) on one side or on both sides were stimulated, usually at a frequency of 0.13 Hz by rectangular pulses of 0.15 ms duration and supramaximal intensity. Sometimes high frequencies up to 200 Hz were used. The isotonic contractions of the ant. tibial muscle(s) were recorded on a smoked drum. OPI (armine or Gd–42, see Table 1), ACh and carbachol were injected through a cannula into the left external jugular vein in a volume of 0.3–0.4 ml. In another series of experiments ACh and carbachol were injected into a femoral artery retrogradely through the cannulated *a. circumflexa femoris lateralis*. The blocking activity of ACh and carbachol was estimated by finding the intra-arterial or intravenous doses that reduced the height of single twitches by 50–60%. To assess the changes in the activity of synaptic ChE, the blocking effects of ACh

Table 1 The ability of organophosphorus compounds to inhibit cholinesterases and to block neuromuscular transmission

	k_{II}† / M^{-1} min^{-1}		*Blocking dose‡*
Inhibitor	*AChE*	*BuChE*	*(mol/kg i.v.)*
 Armine	*	2.6×10^6	$(2.6 \pm 0.2) \times 10^{-6}$ (4)
 Gd–42	2.5×10^8	3.7×10^6	$(3.0 \pm 0.1) \times 10^{-8}$ (5)
 DFP	*	6.7×10^6	

* Not assayed. † k_{II} -bimolecular constants of the rate of interaction of AChE or BuChE with OPI; the k_{II} values for armine and DFP are from Volkova (1965), and the k_{II} values for Gd–42 are from Brestkin, Volkova, Godovikov & Kabachnik (1968). ‡ The dose reducing by 80–90% the height of single twitches of cat ant. tibial muscle; the values are the means ± s.e. mean; the numbers of observations are given in parentheses after the appropriate doses.

and carbachol were compared before and after the administration of OPI. The ability to maintain a tetanus (for 5 s) induced by high frequency stimulation was also tested before and after OPI.

Spontaneous recovery of synaptic transmission impaired by OPI

The contractions of one tibial muscle stimulated at a frequency of 0.13 Hz were recorded. The intra-arterial blocking doses of ACh and carbachol and the ability of the muscle to maintain tetanic contractions were determined before the administration of OPI and at different intervals of time after its administration.

The influence of tetanization on the effect of OPI

The contractions of both tibial muscles stimulated at a frequency of 0.13 Hz were recorded simultaneously. One minute before the injection of OPI the stimulation of the two motor nerves was discontinued. Simultaneously with the intravenous injection of OPI the stimulation of the motor nerve on one side was started and continued for 10 minutes at a frequency of 50–60 Hz. Then, the stimulation of the motor nerves at a frequency of 0.13 Hz was resumed on both sides, and the effects of intravenous injections of ACh and carbachol and the ability to maintain a tetanus were compared in the two muscles.

The isolated phrenic nerve diaphragm preparation of the rat (Bülbring, 1946)

The right and left hemidiaphragms were mounted in two similar baths containing 40 ml Liley solution (Liley, 1956) at 30°C and pH 7.3–7.4 which was bubbled with a gas mixture (95% O_2 and 5% CO_2). The contractions of both hemidiaphragms were recorded in response to stimulation at 0.13 Hz. Drugs were added to the baths in a volume not exceeding 0.5 ml. Before the addition of OPI the stimulation of the two hemidiaphragms was stopped. Simultaneously with the addition of OPI to both baths tetanization of one muscle (50 Hz, for 9 min) was started. OPI remained in the two baths for 7 min and then was washed out three times at 2 min intervals. Tetanization was stopped after the second washing. The stimulation of both muscles at 0.13 Hz was resumed, and the blocking effects of ACh and carbachol (added to the bath for 3 or 5 min), as well as the ability to maintain a tetanus, were compared in the two hemidiaphragms.

Drugs

The following drugs were used: armine, Gd–42 and di*iso*propyl phosphorofluoridate (DFP) (see Table 1), atropine sulphate, acetylcholine chloride (ACh), and carbaminoylcholine chloride (carbachol).

Results

Experiments on cat ant. tibial muscle

The spontaneous recovery of synaptic transmission blocked by OPI is illustrated in Figure 1. Before the administration of OPI, ACh and carbachol given intra-arterially induced a partial block (a reduction of the height of single twitches at a frequency of 0.13 Hz) of about 50% in doses of 10.0 and 0.1 µmol/kg, respectively, and the stimulation of the motor nerve with high frequencies (100–200 Hz, for 5 s) resulted in a well maintained tetanus (Figure 1a). The intravenous injection of OPI (in this case Gd–42, 34 nmol/kg) induced a temporary increase in the height of single twitches and then a complete block of transmission for about 30 min (Figure 1b). After some spontaneous restoration of the contractions (about 70 min after the injection of OPI) the blocking dose of ACh was reduced 2000-fold (0.005 µmol/kg i.a.), but the blocking dose of carbachol remained unchanged (0.1 µmol/kg i.a.) (Figure 1c). At this stage the muscle was unable to maintain contractions even at frequencies of 10 Hz and 35 Hz. When 4 h had elapsed after the injection of OPI (Figure 1d), the blocking dose of ACh had increased 10-fold (0.05 µmol/kg) but the blocking dose of carbachol remained the same (0.1 µmol/kg); at this stage the muscle maintained a tetanus produced by stimulation at 35 Hz, though not at 100 Hz. Similar results were obtained in 17 out of 18 experiments with Gd–42 and in 24 out of 25 experiments with armine. In all cases the blocking dose of ACh was greatly reduced after the administration of OPI, usually 500 to 2000-fold. However, the blocking dose of ACh increased again with time, and when 4–6 h had elapsed after the administration of OPI the blocking dose increased 8 to 15-fold. The blocking dose of carbachol invariably remained the same. The increase with time of the blocking dose of ACh paralleled the increase in the ability of the muscle to maintain a tetanus. These results are consistent with the suggestion that the activity of synaptic ChE, severely inhibited after the injection of OPI, was partly restored in the subsequent few hours and that the gradual improvement of synaptic transmission was due to the recovery of the synaptic ChE activity.

The influence of tetanization of the motor nerve on the effect of OPIs. In previous experiments with armine and Gd–42, it had been shown that the minimal blocking doses of these drugs injected intravenously quickly disappear from the circulating blood, and within 10 min after injection, the OPI cannot be detected in the blood (Ivanov & Muske, 1974). Therefore it seemed reasonable to stimulate the motor nerve for at least 10 min after the injection of OPI to reveal the protective action of the transmitter. Figures 2 and 3 illustrate this form of experiment.

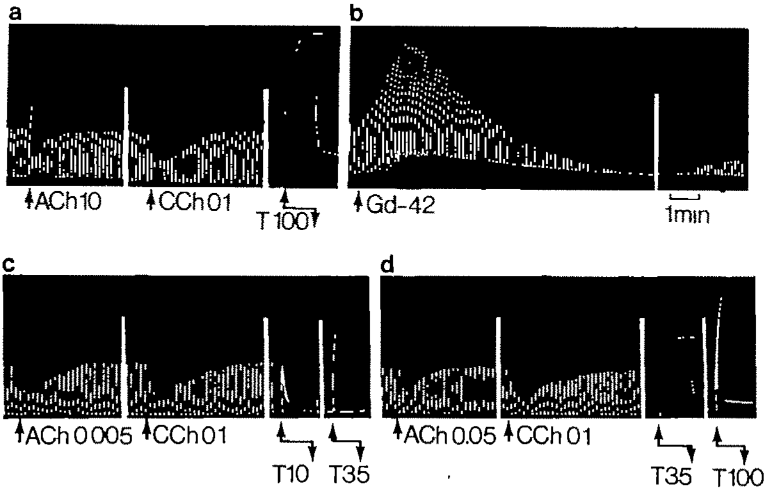


Figure 1 The spontaneous recovery of neuromuscular transmission impaired by organophosphorus cholinesterase inhibitor Gd–42 and the blocking action of intra-arterial injection of acetylcholine (ACh) and carbaminoylcholine (carbachol, CCh). Anaesthetized cat. Atropine 7 mg/kg given intramuscularly. Artificial respiration. Contractions of ant. tibial muscle stimulated indirectly at 0.13 Hz. During periods (5 s) of tetanization (T) at 10–100 Hz the speed of the drum was increased. White vertical lines indicate intervals of 10–15 minutes. (a) Before Gd–42 the blocking doses of ACh and carbachol are 10.0 and 0.1 μmol/kg, respectively. Stimulation at 100 Hz results in a well maintained tetanus. (b) The intravenous injection of Gd–42 34 nmol/kg produces first an increase in the height of contractions and then a complete block of transmission; 30 min later spontaneous restoration of responses begins. (c) Seventy min after the injection of Gd–42; the blocking dose of ACh is reduced 2000-fold (to 0.005 μmol/kg) but the blocking dose of carbachol is unchanged (0.1 μmol/kg). No tetani were obtained at 10 and 35 Hz. (d) Four hours after the injection of Gd–42; the blocking dose of ACh has increased 10-fold (to 0.05 μmol/kg) and the blocking dose of carbachol remains the same (0.1 μmol/kg); stimulation at 35 Hz but not at 100 Hz results in a well maintained tetanus.

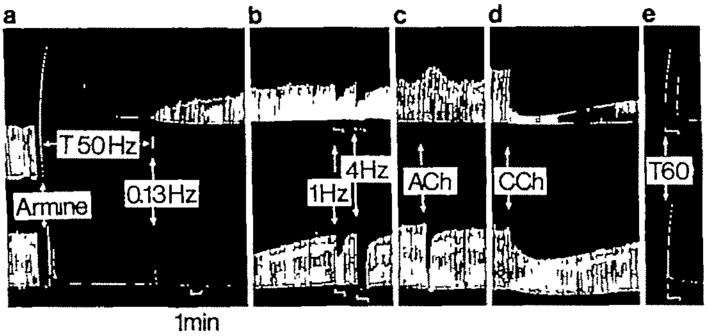


Figure 2 Tetanization of the motor nerve diminishes the impairment of neuromuscular transmission produced by armine. Anaesthetized cat. Atropine 7 mg/kg given intramuscularly. Artificial respiration. Isotonic responses of both ant. tibial muscles induced by indirect stimulation are recorded. White vertical lines—intervals of 20, 10, 20 and 60 min. (a) Stimulation at 0.13 Hz is stopped and armine 2.9 μmol/kg injected intravenously; simultaneously with the injection a 10 min tetanization (T) at 50 Hz of the motor nerve on the left side (upper tracing) is started. During this period the muscle on the control side (lower tracing) shows spontaneous fasciculations induced by armine; after the end of tetanization stimulation at 0.13 Hz is renewed on both sides but induces twitches only on the side of tetanization. (b) Transmission in response to stimuli at 0.13 Hz is restored on the control side but with more frequent stimulation (4 Hz) there is a block on the control side. (c) Acetylcholine (ACh, 0.1 μmol/kg i.v.) produces a blocking effect only on the control side, on the side of tetanization there is a slight increase in the height of contractions which is characteristic of the action of smaller doses of ACh. (d) Carbachol (CCh, 0.5 μmol/kg i.v.) produces a blocking effect on both sides, with that on the side of tetanization somewhat more pronounced. (e) Tetanization at 60 Hz for 5 s (during the tetanization the recording speed was increased); the diminished reaction is much more pronounced on the control side.

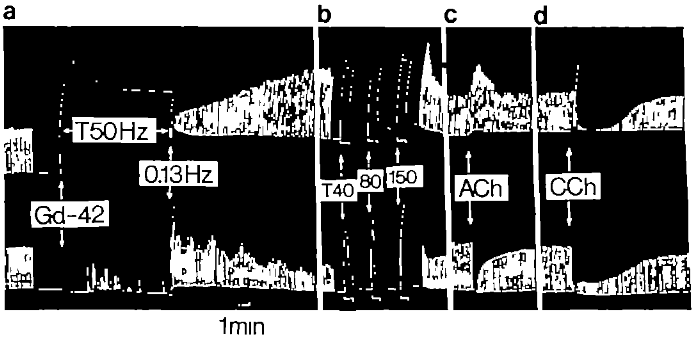


Figure 3 The influence of tetanization on the effect of Gd–42. Cat: Details as in Figure 2. White vertical lines—intervals of 15, 20 and 15 min respectively. (a) Gd–42 (24 nmol/kg i.v.) given simultaneously with the beginning of a 10 min tetanization (T) at 50 Hz on the left side (upper tracing). On the control side (lower tracing) fasciculations of the muscle are seen; after the end of tetanization the stimulation at 0.13 Hz is resumed on both sides causing contractions of increasing size on the side of tetanization and decreasing size on the control side. (b) Tetanization (T) of both motor nerves at 40 Hz, 80 Hz and 150 Hz for 5 s each; on the upper tracing the tetanus is well maintained, but on the control side there is a poor response. (c) Acetylcholine (ACh 0.3 μmol/kg i.v.) induces a complete block of the control muscle and only an increase in the height of the contractions on the side of tetanization. (d) Carbachol (CCh, 0 5 μmol/kg i.v.) induces a block of both muscles more pronounced on the side of tetanization.

Both anterior tibial muscles were stimulated at a frequency of 0.13 Hz. Then stimulation was stopped and armine was injected intravenously (Figure 2a). Simultaneously with the injection of OPI the tetanization of one motor nerve was started and continued for 10 minutes. After the end of tetanization, stimulation at 0.13 Hz was resumed on both sides, but it induced single twitches only on the side of previous tetanization; on the control side, transmission was completely blocked. After the twitches were restored on both sides, stimulation with higher frequencies revealed a marked difference: the muscle previously tetanized maintained a tetanus at 60 Hz but on the control side even 4 Hz caused a complete block (Figure 2e, 2b). The intravenous injection of ACh produced a blocking effect only on the control side, but on the side of tetanization a slight increase in the height of contractions was observed; such an increase is characteristic of the action of smaller (sub-blocking) doses of ACh (Figure 2c). Carbachol induced a block in both muscles and the block was even more pronounced on the side of tetanization (Figure 2d).

Figure 3 illustrates the action of Gd–42. The intravenous injection of ACh induced a complete block of transmission on the control side, but on the side of tetanization only an increase in the height of contractions was observed (Figure 3c). Carbachol induced a block in both muscles and the block was again more pronounced on the side of tetanization (Figure 3d). A tetanus with 40, 80 and 150 Hz was well maintained in the 'protected' muscle but on the control side a tetanus was not maintained (Figure 3b).

Similar results, that is, showing the protective action of tetanization, were obtained in 17 experiments with armine and 14 experiments with Gd–42.

In a control series without administration of OPI (5 experiments), it was shown that tetanization itself (50 Hz, 10 min) did not change either the effects of ACh and carbachol or the reaction of muscle to high frequency tetanization. The protective action of tetanization was also absent when the OPI was injected after the end of tetanization or 10 min before the beginning of tetanization.

The protective action of a tetanization starting simultaneously with the injection of OPI was most obvious when the OPI was injected in a sub-blocking dose (Figure 3a). With a minimal full blocking dose of OPI the protective effect of tetanization was also seen (for instance, Figure 2a), but was usually less pronounced and lasted only 1–1.5 h after the injection of OPI. If OPI was injected in a dose greater than the blocking one the protective effect was usually absent (Figure 4).

Experiments on the isolated rat phrenic nerve diaphragm preparation

Six experiments with Gd–42, 3 with armine and 4 with DFP were performed. Final concentrations of OPI were used that impaired the ability of the muscle to sustain a tetanus at 30–60 Hz after a 7–10 min exposure. These concentrations were 4×10^{-7} M, 1×10^{-6} M and 4×10^{-5} M for Gd–42, armine, and DFP, respectively. One experiment with Gd–42 is illustrated in Figure 5 which shows that the 'protected' muscle maintained a tetanus up to 200 Hz, whereas

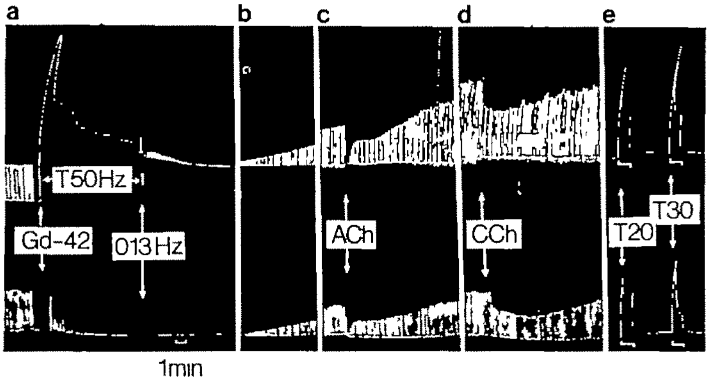


Figure 4 The lack of protective action of tetanization when a large dose of OPI is injected. Cat. Details as in Figure 2. White vertical lines—intervals of 30, 7, 15 and 15 min, respectively. In (a) Gd–42 (50 nmol/kg) is injected intravenously (the minimal full blocking dose is about 34 nmol/kg); a complete block of transmission of single impulses (0.13 Hz) develops both on the tetanized and the control sides. (b) The transmission of single impulses is gradually restored. (c) Acetylcholine (ACh, 40 nmol/kg i.v.) produces a similar blocking effect on the tetanized and on the control muscles. (d) Carbachol (CCh, 0.5 μmol/kg i v.) also induces an equal degree of block of the two muscles. (e) The two muscles react similarly to the tetanization at 20 and 30 Hz (5 s each).

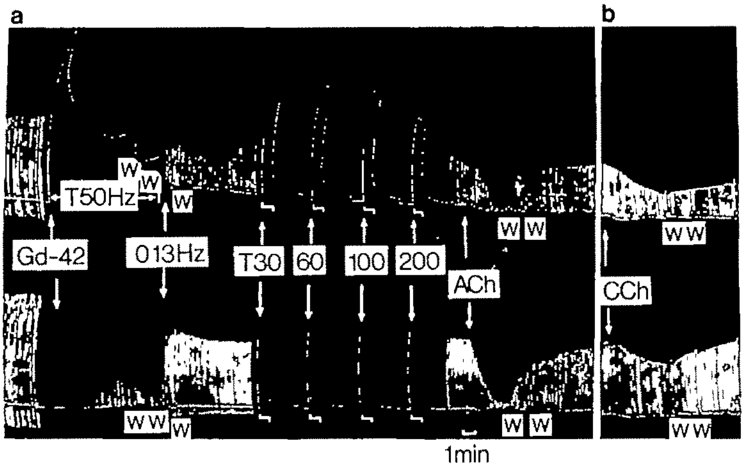


Figure 5 The protective effect of tetanization on the isolated phrenic nerve diaphragm preparation of the rat. The right and left hemidiaphragms are mounted in two similar baths; the isotonic responses to indirect stimulation are recorded. White vertical line—interval of 10 minutes. (a) Stimulation at 0.13 Hz is stopped and Gd–42 (final concentration 4×10^{-7} M) is added to both baths; simultaneously with the addition of OPI tetanization (T) (50 Hz, 9 min) of the left hemidiaphragm (upper tracing) is started; the unstimulated right hemidiaphragm (lower tracing) shows fasciculations. Exposure of both muscles to Gd–42 lasted 7 min, then the inhibitor is washed out 3 times with an interval of 2 min, the tetanization is stopped and the stimulation of the two muscles at 0.13 Hz resumed. With short (6 s) tetanizations (T) at 30, 60, 100 and 200 Hz the upper muscle maintains all contractions well whereas in the lower muscle no tetanus is maintained even with 30 Hz. Acetylcholine (ACh, 3×10^{-5} M, 3 min) produces a more pronounced block on the control (lower) muscle than on the tetanized muscle. (b) Carbachol (CCh, 4×10^{-5} M, 5 min) produces a similar effect on both muscles.

the control muscle did not maintain a tetanus with 30 Hz (Figure 5a). The addition of ACh (final concentration 4×10^{-5} M) produced a blocking effect on both muscles but on the control muscle the block was more pronounced (Figure 5a). With carbachol the block was nearly equal on both sides (Figure 5b). Similar results were obtained in other experiments. In two experiments with DFP the protective effect of tetanization was very weak. Generally the protective effect of tetanization on the rat diaphragm *in vitro* was less pronounced than in the cat ant. tibialis *in situ*.

Discussion

Tetanization of the motor nerve, started simultaneously with the intravenous injection of OPI, diminished the impairment of neuromuscular transmission and presumably the degree of synaptic ChE inhibition produced by OPI.

It has been shown in rats that a prolonged (1–2 h) tetanization of the motor nerve increased the activity of synaptic ChE (histochemical determination) but the total ChE activity of the muscle (biochemical determination) did not change (Gerebtzoff, Goffart & Dresse, 1963). The authors suggested that during the prolonged tetanization the enzyme moved from the sarcoplasm into the synapse. This suggested mechanism cannot explain our results because in the absence of OPI the 10 min tetanization did not change the blocking action of ACh.

Our results can be explained on the theory that ACh at the synapse can protect a part of the synaptic ChE from irreversible inhibition by OPI and that the increase in the liberation of ACh during tetanization enhances this protective effect. Unfortunately we do not know the concentration of transmitter that can be achieved in the synaptic cleft after the inactivation of synaptic ChE. Nevertheless this concentration is probably high enough for a protective effect. The ACh concentration in nerve terminal vesicles is very high, not less than 1.5×10^{-1} M in warm blooded animals. Even after dilution of this concentration by several magnitudes the ability to protect ChE from irreversible inhibition should still be retained (Koelle, 1946; Brestkin *et al.*, 1964; Volkova, 1965).

Biochemical determinations of residual ChE activity in cat muscle homogenates revealed that on the side of tetanization the inhibition was usually somewhat less pronounced than on the control side (Ivanov *et al.*, 1972). But the main proof of a protective effect of tetanization was obtained by comparison of the effects of ACh and carbachol on the two sides. By this method it was possible to reveal a protection of the synaptic ChE, which is the only portion that is important for synaptic transmission.

The protective action of ACh cannot be achieved when the enzyme is already phosphorylated by OPI. Thus tetanization started 10–20 min after the injection of OPI had no protective action. When tetanization was finished before the injection of OPI the impairment of transmission could even be increased, probably because of the increased supply to the muscle of OPI due to post-tetanic hyperaemia.

The protective effect of tetanization diminished with an increase in the dose of OPI, and was sometimes absent with very large doses. One possible reason is that with a large dose, OPI does not disappear from the blood during the period of tetanization (10 minutes). The protective effect also diminished with a minimal blocking dose if the tetanization lasted only 1–3 minutes. Furthermore, with high doses of OPI the irreversible inhibition of synaptic ChE may occur before the accumulation of ACh in a concentration sufficient for effective protection.

It has been shown that in rats the spontaneous restoration of neuromuscular transmission blocked with sarin is accelerated by raising the muscle temperature by 3–4 °C (Meeter & Wolthuis, 1968). In our experiments on cats only a slight (0.6–1.6 °C) rise of temperature in the tetanized muscle was observed. The temperature of the control muscle did not change significantly after the administration of OPI. However, the observed small rise of temperature cannot explain the protective effect of tetanization because such a rise produced by artificial heating of the leg (instead of by tetanization) did not influence the effect of OPI (Ivanov, 1973).

Data have been published (Khayutin, 1971) indicating that during a strong tetanic contraction the blood vessels can be constricted and the blood supply to the muscle impaired. If so, the reduction of the blood supply of the tetanized muscle would reduce the amount of OPI reaching the muscle and this could be the major reason for the protective effect of tetanization. But control experiments showed that the blood flow through the femoral artery was not reduced but markedly increased on the side of tetanization started simultaneously with the injection of OPI, and that it greatly surpassed the blood flow on the control side (Ivanov *et al.*, 1972). Thus, the protective effect of tetanization occurred in spite of the fact that the amount of OPI delivered to the side of tetanization was probably greater.

The protective effect of tetanization may be due not only to the action of transmitter ACh, as we have suggested earlier (Ivanov *et al.*, 1972). In experiments on the longitudinal muscle of the guinea-pig ileum, Mittag, Ehrenpreis & Patrick (1971) have shown that ACh added to the bath is hydrolysed 4 times less rapidly in isotonic conditions, when the muscle is shortened under the influence of ACh, than in isometric conditions when the muscle remains in the extended state. The authors suggested that the penetration of ACh to the muscle ChE is hampered when the muscle is contracted and shortened.

Michelson & Shelkovnikov (1976) have presented evidence that the isolated rectus abdominis muscle of the frog also hydrolyses ACh more rapidly in an extended state (isometric conditions) than in a shortened state (isotonic recording).

Lancaster (1973) added a quaternary ammonium OPI to the bath containing a nerve-muscle preparation of rat diaphragm and observed that in the tetanized muscle the degree of ChE inhibition was less than in the control.

In the light of all these findings one can postulate that the shortening of the muscle contracting under isotonic conditions impedes the penetration to muscle ChE not only of substrates but also of anticholinesterases. We recorded the responses isotonically and the protective effect of tetanization could have been due partly to the hampered penetration of OPI to the ChE of the tetanized muscle in spite of its better blood supply. Indeed, the corresponding control experiments (5 trials) had shown that the protective effect of tetanization is less pronounced in isometric than in isotonic conditions. In another series (7 experiments) both muscles were tetanized but during the tetanization one muscle was under isotonic and the other under isometric conditions. In 4 cases the protectice effect of tetanization was more pronounced on the side of isotonic responses (showing the protective effect of shortening). In 3 other experiments the effect of OPI was similar on both muscles. Thus, in isotonic conditions the protective effect of tetanization is probably due not only to the increased protection of synaptic ChE by the transmitter, but also to the greater difficulty of penetration of the OPI in the tetanized (and shortened) muscle.

Our experiments have shown that the spontaneous restoration of neuromuscular transmission blocked by OPI is probably accompanied by an increase in the residual activity of synaptic ChE. This is indicated by the gradual decrease in the blocking action of ACh during the first hours after the injection of OPI (Figure 1). It is unlikely that the increase in the blocking doses of ACh is due to desensitization of cholinoreceptors because the blocking dose of carbachol did not change.

It is clear that ACh released in the synapse during the development of the ChE inhibition by OPI not only protects a part of the synaptic ChE from irreversible inhibition but also inhibits a part of the protected synaptic AChE by the mechanism of substrate inhibition. Substrate inhibition requires higher concentrations of ACh (about 10^{-2} M) than those required for the protective effect. Nevertheless there is some evidence that the local concentration of ACh can be sufficient for transient substrate inhibition even in the absence of OPI (Cohen & Hagen, 1964; Kerkut, Geoffrey, Rick & Walker, 1970; Whittaker & Dowdall, 1975). In this case the block of transmission must be due to heterogeneous inhibition of the synaptic enzyme: one part (probably the greater) will be irreversibly inhibited by OPI, and another part will be under reversible inhibition by ACh. Some decrease in the concentration of ACh in the synaptic cleft will be sufficient for reactivation of the latter portion of AChE. OPI quickly disappears from the blood (Ivanov & Muske, 1974) and the reactivated part of the enzyme will not be inactivated again by OPI. Even if only a small part of synaptic AChE was under substrate inhibition its reactivation can significantly improve transmission, an increase of a few percent may be enough (Barstad, 1960; Barnard, Wieckowski & Chin, 1971). However, the substrate inhibition probably disappears fairly quickly and therefore it seems more reasonable to ascribe the increase in synaptic AChE activity occurring in 4–6 h after the administration of OPI mainly to the synthesis of new enzyme or to partial reactivation of the phosphorylated enzyme.

We should like to thank Dr G.B. Koelle and Dr Marthe Vogt for helpful suggestions and discussions.

References

ALDRIDGE, W.N. (1950). Some properties of specific cholinesterase with particular reference to the mechanism of inhibition by diethyl p-nitrophenyl thiophosphate (E 605) and analogues. *Biochem. J.*, **46**, 451–460.

AUGUSTINSSON, K.B. & NACHMANSOHN, D. (1949). Studies on cholinesterase; kinetics of the inhibition of acetylcholine esterase. *J. biol. Chem.*, **179**, 543–559.

BARNARD, E.A., WIECKOWSKI, J. & CHIN, T.H. (1971). Cholinergic receptor molecules and cholinesterase molecules at mouse skeletal muscle junction. *Nature, Lond.*, **234**, 207–209.

BARSTAD, J.A.B. (1960). Cholinesterase inhibition and the effect of anticholinesterases on indirectly evoked single and tetanic muscle contractions in the phrenic nerve-diaphragm preparation from the rat. *Arch. int. Pharmacodyn. Thér.*, **128**, 143–168.

BERRY, W.K. & EVANS, C.L. (1951). Cholinesterase and neuromuscular block. *J. Physiol*, **115**, 46–47P.

BOWMAN, W.C. & HEMSWORTH, B.A. (1965). Effects of triethylcholine on the output of acetylcholine from the isolated diaphragm of the rat. *Br. J. Pharmac. Chemother.*, **24**, 110–118.

BRESTKIN, A.P., VOLKOVA, R.I., GODOVIKOV, N.N. & KABACHNIK, M.I. (1968). Anticholinesterase properties of O-ethyl-S-(β-alkylmercapto-ethyl) methylthiophosphonates and their methylsulphomethylates. *Izv. Akad. Nauk SSSR, ser. Khim.*, **9**, 2028–2032 (in Russian).

BRESTKIN, A.P., VOLKOVA, R.I. & ROSENGART, E.V. (1964). On the protective action of acetylcholine during the interaction of organophosphorus inhibitors with serum cholinesterase. *Proc. Acad. Sci. USSR (Dokl. Akad. Nauk SSSR)*, **157**, 1459–1462 (in Russian).

BÜLBRING, E. (1946). Observations on the isolated phrenic nerve diaphragm preparation of the rat. *Br. J. Pharmac. Chemother.*, **1**, 38–61.

BURGEN, A.S.V. (1949). Mechanism of action of anticholinesterase drugs. *Br. J. Pharmac. Chemother.*, **4**, 219–228.

COHEN, L.H. & HAGEN, P.B. (1964). A physiological role for the presynaptic localization of acetylcholinesterase and for its inhibition by excess substrate *Can. J. Physiol. Pharmac.*, **42**, 593–594.

DANILOV, A.F. (1967). Inhibition of the myoneural synapses cholinesterase mediated through decamethonium and ditilin (celocaine). *Pharmac. Toxicol., Moscow*, **30**, 664–669 (in Russian).

DANILOV, A.F. & IVANOV, YU.J. (1972). The mechanism of blockade and recovery of the neuromuscular transmission produced by armine in cats. *Pharmac. Toxicol., Moscow*, **35**, 571–575 (in Russian).

GEREBTZOFF, M.A., GOFFART, M. & DRESSE, A. (1963). Effet de la stimulation motrice sur l'activité acétylcholinestérasique à la jonction myoneurale. *Compt. rend. Soc. Biol.*, **157**, 420–422.

IVANOV, YU.J. (1972). On the role of substrate inhibition of synaptic acetylcholinesterase in the mechanism of action of irreversible cholinesterase inhibitors on the neuromuscular transmission. *Proc. Acad. Sci. USSR (Dokl. Akad. Nauk SSSR)*, **202**, 1464–1466 (in Russian).

IVANOV, YU.J. (1973). On the mechanism of the blocking action of organophosphorus cholinesterase inhibitors on the neuromuscular transmission. *Thesis, Institute of Experimental Medicine, Leningrad* (in Russian).

IVANOV, YU.J., DANILOV, A.F., ZELIKSON, B.B. & MICHELSON, M.J. (1972). The influence of tetanization of the skeletal muscle motor nerve on the blocking and anticholinesterase effects of armine and the Gd–42 compound. *Pharmac. Toxicol., Moscow*, **35**, 567–570 (in Russian).

IVANOV, YU.J. & MUSKE, G.A. (1974). Circulation time of reacting armine in the blood stream of a cat after intravenous administration of the drug. *Pharmac. Toxicol., Moscow*, **37**, 297–300 (in Russian).

KERKUT, G.A., GEOFFREY, W.O., OLIVER, RICK J.T. & WALKER, R.J. (1970). The effects of drugs on learning in a simple preparation. *Comp. gen. Pharmac.*, **1**, 437–483.

KHAJUTIN, V.M. (1971). The mechanisms of regulation of the vessels tonus in a working muscle. A histomechanical hypothesis. In *Problems of modern physiology*, ed. Kreps, E.M. & Chernigovsky, V.N., pp. 123–140. Leningrad: Nauka (in Russian).

KOELLE, G.B. (1946). Protection of cholinesterase against irreversible inactivation by di-isopropylfluorphosphate *in vitro*. *J. Pharmac. exp. Ther.*, **88**, 232–237.

KOELLE, G.B. (1963). Cytological distributions and physiological functions of cholinesterases. In *Cholinesterases and anticholinesterase agents, Handbuch der experimentellen Pharmakologie*, Bd. **15**, ed. Koelle, G.B., pp. 187–299. Berlin: Springer-Verlag.

KRNJEVIĆ, K. & MITCHELL, J.F. (1961). The release of acetylcholine in the isolated rat diaphragm. *J. Physiol.*, **155**, 246–262.

LANCASTER, R. (1973). Relationships between *in vivo* and *in vitro* inhibition of acetylcholinesterase (AChE) and impairment of neuromuscular transmission in the rat phrenic-nerve diaphragm by a tertiary anticholinesterase and its quaternary analogue. *Biochem. Pharmac.*, **22**, 1875–1881.

LILEY, A.W. (1956). An investigation of spontaneous activity at the neuromuscular junction of the rat. *J. Physiol.*, **132**, 650–666.

MCNAMARA, B.P., MURTHA, E.F., BERGNER, A.D., ROBINSON, E.M., BENDER, C.W. & WILLS, J.H. (1954). Studies on the mechanism of action of DFP and TEPP. *J. Pharmac. exp. Ther.*, **110**, 232–240.

MEETER, E. (1969). Desensitization of the end-plate membrane following cholinesterase inhibition an adjustment to a new working situation. *Acta Physiol. Pharmac. Neerl.*, **15**, 243–258.

MEETER, E. & WOLTHUIS, O.L. (1968). The spontaneous recovery of respiration and neuromuscular transmission in the rat after anticholinesterase poisoning. *Eur. J. Pharmac.*, **2**, 377–386.

MICHELSON, M.J., MUSKE, G.A. & PROTAS, L.L. (1974). On the role of blood and muscle cholinesterases in the hydrolysis of subecholine and acetylcholine in the cat. *Pharm. Res. Comm.*, **6**, 281–288.

MICHELSON, M.J. & SHELKOVNIKOV, S.A. (1976). Isotonic and isometric responses of different tonic muscles to agonists and antagonists. *Br. J. Pharmac.* (in press).

MITTAG, T.W., EHRENPREIS, S. & HEHIR, R.M. (1971). Functional acetylcholinesterase of rat diaphragm muscle. *Biochem. Pharmac.*, **20**, 2263–2273.

MITTAG, T.W., EHRENPREIS, S. & PATRICK, P. (1971). Some properties of cholinesterases in intact guinea-pig ileum *in vitro*. *Arch. int. Pharmacodyn.*, **191**, 270–278.

OSTROWSKI, K. & BARNARD, E.A. (1961). Application of isotopically-labelled specific inhibitors as a method in enzyme cytochemistry. *Expl Cell Res.*, **25**, 465–468.

STRAUGHAN, D.W. (1960). The release of acetylcholine from mammalian motor nerve endings. *Br. J. Pharmac.*, **15**, 417–424.

VOLKOVA, R.I. (1965). On the interaction of cholinesterase with irreversible organophosphorus inhibitors in the presence of substrate. *Biochem., Moscow*, **30**, 292–301 (in Russian).

WHITTAKER, V.P. & DOWDALL, M.J. (1975). Current state of research on cholinergic synapses. In *Cholinergic Mechanisms*, ed. Waser, P.G., pp. 23–42. New York: Raven Press.

(Received September 12, 1975)

METABOLISM OF PHENYLETHYLAMINE IN RAT ISOLATED PERFUSED LUNG: EVIDENCE FOR MONOAMINE OXIDASE 'TYPE B' IN LUNG

Y.S. BAKHLE
Department of Pharmacology, Institute of Basic Medical Sciences,
Royal College of Surgeons of England, Lincoln's Inn Fields, London WC2A 3PN
M.B.H. YOUDIM
MRC Unit and University Department of Clinical Pharmacology, Radcliffe Infirmary,
Oxford OX2 6HE

Phenylethylamine is inactivated in a single passage through rat lung tissue by a process of uptake and deamination by a monoamine oxidase 'type B'. This enzyme is particularly susceptible to inhibition by deprenil and less sensitive to clorgyline. The monoamine oxidase of the lung, like that of other rat tissues, can be differentiated into 'type A' and 'type B' which appear to operate independently in the organized tissue.

Introduction Monoamine oxidase (MAO) has been differentiated into MAO 'type A' (MAO-A) and 'type B' (MAO-B) on the basis of substrate and inhibitor specificity: noradrenaline and 5-hydroxytryptamine (5-HT) are substrates and clorgyline an inhibitor for MAO-A (Johnston, 1968), whereas phenylethylamine and benzylamine are substrates and deprenil an inhibitor for MAO-B (Yang & Neff, 1973; Knoll & Magyar, 1972). Although MAO-B is present in tissue homogenates, its presence in organized tissue has not yet been demonstrated (Bakhle & Vane, 1974; Youdim & Woods, 1975). The experiments to be described are the first demonstration† of MAO-B activity in an isolated perfused tissue. We have used the rat isolated lung perfused with Krebs solution since substrates for MAO-A, 5-HT and noradrenaline, are metabolized on a single passage through the pulmonary circulation (for references see Bakhle & Vane, 1974) and MAO-B activity is present in homogenates of rabbit lung (Roth & Gillis, 1974) and of rat lung (Bakhle & Youdim, unpublished experiments). By studying the fate of phenylethylamine and comparing it with that of 5-HT in rat isolated perfused lung, we hoped to demonstrate the presence of MAO-A and B and to explore the interactions between the metabolism of these two amines in the whole tissue. Since metabolism by MAO reduces the biological activity of these amines we have followed the action of MAO by bioassay as well as by radiochemical assay.

Methods The isolated lung was perfused via the pulmonary artery with warm (37°C), oxygenated Krebs solution at 8 ml/minute. For bioassay, the effluent from the lung superfused rat stomach strips (Vane, 1957) which respond to both amines with contractions, although phenylethylamine is about 1000 times less potent than 5-HT. On this tissue, phenylethylamine acts via tryptamine receptors (Vane, 1960). For the radiochemical assay we collected the effluent in 0.5 min fractions during and after the infusion of radioactive amines. In each fraction we measured the total radioactivity and separated this radioactivity into basic and non-basic materials by ion exchange chromatography (Southgate & Collins, 1969). The radioactivity associated with the non-basic material was taken to represent the metabolite(s) of 5-HT and of phenylethylamine.

Results and Discussion These experiments showed that 75% of infused radioactive phenylethylamine had emerged from the lung within 5 min, i.e. 1 min after the end of the infusion (0–4 min) and that after 30 min, 90% had left the lung. The radioactivity associated with non-basic material, i.e. metabolite of phenylethylamine, had a maximum value during the infusion of about 40% of the total but after about 6 min, nearly all radioactivity in the effluent was metabolite. The high proportion of administered radioactivity in the lung effluent and the rapidity with which it appeared, argue strongly against the binding of amine in a store, e.g. nerve granules. The same two characteristics have been observed for the removal of 5-HT in rat lung (Alabaster & Bakhle, 1970).

At the concentration used, the biological inactivation of phenylethylamine was between 50 and 60%. The radiochemical assay showed that about 55% of the infused radioactivity which emerged from the lung was metabolite. We feel that the correspondence between these two values is close enough to conclude that the loss of biological activity observed when phenylethylamine passes through the rat isolated lung is due chiefly to its metabolism by a monoamine oxidase type of enzyme.

Since phenylethylamine is the substrate, this monoamine oxidase should be of the B type and thus more susceptible to inhibition by deprenil than by clorgyline (Johnston, 1968; Knoll & Magyar, 1972). Experiments with these two monoamine oxidase inhibitors substantiated this prediction and are summarized in Table 1.

All the earlier work with 5-HT and noradrenaline had emphasized the importance of the uptake step in the overall inactivation process and the question of competition between noradrenaline and 5-HT for uptake has received some attention (Junod, 1972; Alabaster & Bakhle, 1973; Iwasawa & Gillis, 1974; Nicholas, Strum, Angelo & Junod, 1974). We looked for competition between 5-HT and phenylethylamine uptake by measuring metabolism of one labelled substrate in presence of the other. Competition for uptake should show itself as decreased metabolism. Our experiments are summarized in Table 1 and show that there was no competition between the amines at equimolar concentrations and even when phenylethylamine was present at almost 200 times molar excess, the metabolism of 5-HT was not inhibited.

An interesting point to emerge from Table 1 is that at equimolar concentrations (1.25×10^{-5} M), phenylethylamine suffered about 50% metabolism, whereas 5-HT was less affected. Clearly the removal process for 5-HT is close to its limit whereas that for phenylethylamine seems to be well below its limit, at 1.25×10^{-5} M. For comparison, the *Km* values for the A and B type enzymes isolated from liver are 18.7×10^{-5} M and 2.0×10^{-5} M with 5-HT and phenylethylamine as substrates respectively (Houslay & Tipton, 1974). On the basis of these *Km* values neither enzyme would be expected to be saturated at concentration of substrates of 1.25×10^{-5} M; therefore either the lung enzymes have very different *Km* values from the liver enzymes, or in the perfused lung the uptake of amines into lung cells is limiting. We favour the latter possibility. The saturation of amine uptake in lung has been noticed earlier (Junod, 1972; Hughes, Gillis & Bloom, 1969); for 5-HT in rat lung the saturating concentrations should be about 10 µM (Junod, 1972) and our results would agree with this. Whether or not there is biological significance in the different capacities of the lung removal processes for 5-HT and phenylethylamine is a matter for speculation. What is more open to investigation is the difference in the uptake mechanisms for the two amines.

In conclusion these experiments strengthen our view (Bakhle & Vane, 1974; Youdim & Woods, 1975) that the enzymatic activity exhibited by the whole tissue is often strikingly different from that found in cell-free extracts. For instance, in partially purified preparations of MAO, 5-HT and phenylethylamine will compete for the enzyme site (Houslay & Tipton, 1975). This demonstration of both A and B type monoamine oxidases operating independently in an organized tissue makes it more likely that we shall find these two enzymatic activities playing a real and independent role *in vivo*, not only in lung but also in other tissues such as brain.

This work was supported by the M.R.C.

† *Note added in proof.* While this paper was in press, essentially similar findings in rabbit lungs have been reported by Roth & Gillis (1975) 'Multiple forms of amine oxidase in perfused rabbit lung'. *J. Pharmac. exp. Ther.*, **194**, 537–544.

Table 1 Effect of monoamine oxidase inhibitors on [^{14}C]-phenylethylamine deamination and metabolism of phenylethylamine and 5-hydroxytryptamine in presence of each other during transit through rat isolated lung

Labelled amine (final conc in perfusion fluid)	*No. of experiments*	*Inhibitor*	*Competing amine*	*Radioactivity associated with metabolite(s) (% of total)**	*% Inhibition of metabolism*
[^{14}C]-Phenylethylamine ($1\ 25 \times 10^{-5}$ M)	8	None	None (control)	55.8 ± 3 6	
	5	Deprenil	None	17 4 ± 2 5	69
	3	Clorgyline	None	40.2 ± 1.5	28
	3	None	5-hydroxytryptamine ($1\ 25 \times 10^{-5}$ M)	54.7 ± 0.3	2
[^{3}H]-5-Hydroxytryptamine (1.25×10^{-5} M)	4	None	None (control)	11.6 ± 1 3	–
	2	None	Phenylethylamine (1.25×10^{-5} M)	11.0 ± 12.3	0
[^{3}H]-5-Hydroxytryptamine (7×10^{-8} M)	7	None	None (control	53 6 ± 2 6	–
	4	None	Phenylethylamine (1.25×10^{-5} M)	53.8 ± 4 1	0

The inhibitors deprenil or clorgyline were infused for 10 min before the infusion of phenylethylamine (4 min) and throughout the subsequent collection period (30 minutes) The concentration of these two inhibitors was 100 µg/ml in each case. The labelled amines [1-^{14}C]-phenylethylamine or [G-^{3}H]-5-hydroxytryptamine (approximately 60,000 d/min) were infused for 4 minutes. The competing amine, unlabelled, was infused for 5 min before, during and for 25 min after, the infusion of labelled amine.

* Mean ± s.e. mean.

References

ALABASTER, V.A. & BAKHLE, Y.S. (1970). Removal of 5-hydroxytryptamine in the pulmonary circulation of rat isolated lungs. *Br. J. Pharmac.*, **40**, 468–482.

ALABASTER, V.A. & BAKHLE, Y.S. (1973). The removal of noradrenaline in the pulmonary circulation of rat isolated lungs. *Br. J. Pharmac.*, **47**, 325–331.

BAKHLE, Y.S. & VANE, J.R. (1974). Pharmacokinetic function of the pulmonary circulation. *Physiol. Rev.*, **54**, 1007–1045.

HOUSLAY, M.D. & TIPTON, K.F. (1974). A kinetic evaluation of monoamine oxidase activity in rat liver mitochondrial outer membranes. *Biochem. J.*, **139**, 645–652.

HOUSLAY, M.D. & TIPTON, K.F (1975). Amine competition for oxidation by rat liver mitochondrial monoamine oxidase. *Biochem. Pharmac.*, **24**, 627–631.

HUGHES, J., GILLIS, C.N. & BLOOM, F.E (1969). The uptake and disposition of dl-norepinephrine in perfused rat lungs. *J. Pharmac. exp. Ther.*, **169**, 237–248.

IWASAWA, Y & GILLIS, C.N. (1974). Pharmacological analysis of norepinephrine and 5-hydroxytryptamine removal from the pulmonary circulation: differentiation of uptake sites for each amine. *J. Pharmac. exp. Ther.*, **188**, 386–393.

JOHNSTON, J.P. (1968). Some observations upon a new inhibitor of monoamine oxidase in brain tissue. *Biochem. Pharmac.*, **17**, 1285–1297.

JUNOD, A.F. (1972). Uptake, metabolism and efflux of ^{14}C-5-hydroxytryptamine in isolated perfused lungs. *J. Pharmac. exp. Ther.*, **183**, 341–355.

KNOLL, J & MAGYAR, K (1972). Some puzzling pharmacological effects of monoamine oxidase inhibitors. *Adv. Biochem. Psychopharmac.*, **5**, 393–408.

NICHOLAS, T.E., STRUM, J.M., ANGELO, L.S. & JUNOD, A.F. (1974). Site and mechanism of uptake of 3-H-norepinephrine by isolated perfused rat lungs. *Circulation Res.*, **35**, 670–680.

ROTH, J.A. & GILLIS, C.N. (1974). Deamination of β-phenylethylamine by monoamine oxidase-inhibition by imipramine. *Biochem. Pharmac.*, **23**, 2537–2545.

SOUTHGATE, J. & COLLINS, G.G.S. (1969). The estimation of monoamine oxidase using ^{14}C-labelled substrates. *Biochem. Pharmac.*, **18**, 2285–2287.

VANE, J.R. (1957). A sensitive method for the assay of 5-hydroxytryptamine. *Br. J. Pharmac. Chemother.*, **12**, 344–349.

VANE, J.R. (1960). In *Adrenergic mechanisms*, ed. Vane, J.R., Wolstenholme, G.E.W. & O'Connor, M., pp. 356–373. London: Churchill.

YANG, H.Y.T. & NEFF, N.H. (1973). β-Phenylethylamine: a specific substrate for type B monoamine oxidase of brain. *J. Pharmac. exp. Ther.*, **187**, 365–371.

YOUDIM, M.B.H. & WOODS, H.F. (1975). The influence of tissue environment on the rates of metabolic processes and the properties of enzymes. *Biochem. Pharmac.*, **24**, 317–323.

(Received September 4, 1975)

Br. J. Pharmac. (1976), 56, 128–130

A NOTE ON THE EFFECT OF DITHIOTHREITOL (DTT) ON THE DEPOLARIZATION OF ISOLATED SYMPATHETIC GANGLIA BY CARBACHOL AND BROMO-ACETYLCHOLINE

D.A. BROWN & D. KWIATKOWSKI

Department of Pharmacology, The School of Pharmacy, University of London,
29/39 Brunswick Square, London WC1N 1AX

The S–S reducing agent, dithiothreitol (DTT) altered the properties of nicotinic receptors in rat superior cervical ganglia such that (i) carbachol became less active as a depolarizing agent and (ii) bromo-acetylcholine produced an irreversible depolarization. The latter was temporarily annulled by hexamethonium (which retained antagonist properties), but returned when hexamethonium was removed. It is concluded that ganglionic nicotinic receptors might be quite similar to those for mono-quaternary agonists in leech dorsal muscle.

Introduction Reduction of disulphide bonds to free sulphydryl (–SH) groups with dithiothreitol (Cleland, 1964) has some interesting effects on the responses of electroplax and striated muscle to acetylcholine-receptor ligands. Reported effects include: (i) reduced affinity of acetylcholine and carbachol; (ii) generation of irreversible agonist activity with halogenated acetylcholine compounds; and (iii) generation of an agonist response to hexamethonium (Karlin & Bartels, 1966; Karlin & Winnik, 1968; Albuquerque, Sokoll, Sonneson & Thesleff, 1968; Mittag & Tormoy, 1970; Rang & Ritter, 1971; Ross & Triggle, 1972; Ben-Haim, Landau & Silman, 1973; Ben-Haim, Dreyer & Peper, 1975).

The objective of the present experiments was to find out whether ganglionic nicotinic receptors showed any or all of these changes after S–S reduction with DTT, as an aid to the eventual definition of their relationship with other nicotinic receptors.

Methods Demarcation potentials between ganglion and postganglionic nerve trunk were recorded from isolated superior cervical ganglia of the rat with extracellular electrodes, using a continuous superfusion technique (Brown & Marsh, 1975). The superfusion fluid consistent of Krebs solution at 25°C containing hyoscine 1 μM (to annul effects on muscarinic receptors) and physostigmine 10 μM (to prevent hydrolysis of bromo-acetylcholine; Chiou & Rama Sastry, 1968). The normal pH of the bicarbonate-buffered solution bubbled with 95% O_2 and 5% Co_2 was 7.4; during application of DTT a Tris-buffered solution at pH 8 was used.

Drugs. Dithiothreitol (DTT) and 5′5-dithio-*bis* (2-nitrobenzoic acid) (DTNB) were obtained from Sigma. Bromo-acetylcholine (BAC) was synthesized and purified as the perchlorate salt according to the method of Chiou & Rama Sastry (1968); a second sample was kindly provided by Professor Chiou.

Results

Action of bromoacetylcholine In the normal ganglion (i.e. without prior S–S reduction) BAC produced a readily-reversible depolarization; in contrast to the observation of Chiou (1974) on skeletal muscle, no evidence for irreversibility was obtained after contact times up to 20 minutes. Compared with carbachol, BAC was (i) slightly (1.5–2 times) more effective (in the presence of physostigmine) and (ii) tended to show greater desensitization (Figure 1).

Effect of dithiothreitol DTT was applied for 15 min at pH 8, and was then washed out for 10 min with normal Krebs solution at pH 7.4 before testing the agonist. DTT itself produced small and inconsistent changes in the resting demarcation potentials.

(a) Carbachol—DTT (1 mM) shifted the carbachol dose-response curve to the right by some 0.8 log units; subsequent exposure to 3 mM DTT produced a slight further shift. This depression persisted for several hours after DTT, and was fully reversed by 10 min reoxidation with 1 mM DTNB.

(b) BAC—After DTT, the depolarization produced by BAC became irreversible, i.e. polarity was not restored on washing out the BAC with Krebs solution (Figure 1). Depolarization was reversed temporarily by hexamethonium, but returned on washing out the hexamethonium. Even after overnight washing in Krebs solution after a single 2 min application of BAC, addition of hexamethonium still produced a hyperpolarization, suggesting that the depolarization was very persistent. With continuous recording the level of depolarization tended to diminish gradually, but this might have been due to spread of depolarization to the reference electrode on the postganglionic nerve trunk, consequent upon a discharge of the ionic gradients (cf. Brown,

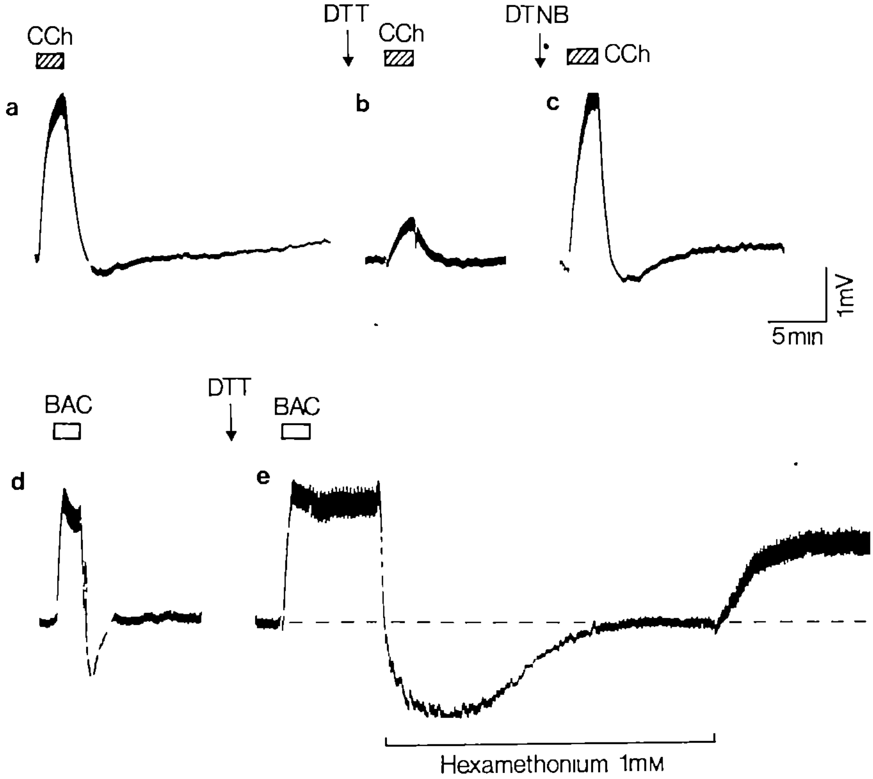


Figure 1 Effects of dithiothreitol (DTT, 1 mM, added for 15 min at the arrows and then washed out) on depolarizing responses to carbachol (CCh, 55 μM) and bromo-acetylcholine (BAC, 32 μM) recorded potentiometrically. The reversible response to carbachol (a) was reduced after DTT (b) and restored after 10 min application of 5′5-dithio-*bis*-(2-nitrobenzoic acid) (DTNB) (c). After DTT (e), the depolarization produced by BAC which was normally reversible (d) persisted when the BAC was washed out, was reversed on addition of hexamethonium 1 mM, but reappeared when the hexamethonium was washed out. The post-agonist hyperpolarizations are due to electrogenic extrusion of accumulated Na^+ ion (Brown *et al.*, 1972) This is very pronounced on addition of hexamethonium during post-DTT BAC-depolarization; the projected baseline demarcation potential in the absence of electrogenic hyperpolarization is indicated by the dashed line. Agonists were added for 2 min at 30 min intervals. Scales: 1 mV, 5 minutes

Brownstein & Scholfield, 1972; Brown & Scholfield, 1974). In Figure 1, the reversal of potential during exposure to hexamethonium is due to the electrogenic extrusion of accumulated Na^+ ion, see Brown *et al.* (1972); the very prolonged hyperpolarization after BAC would reflect the large Na^+ accumulation during the irreversible depolarization.

Three other features of post-DTT BAC depolarization may be noted. (i) The desensitization was much less pronounced. (ii) The magnitude of the depolarization was still dependent upon the concentration of BAC, such that step-wise increments in the depolarization occurred with cumulative addition of BAC. The sensitivity of the preparation to BAC, as judged from these increments, appeared to be greater after DTT, although the peak magnitude of the depolarization was usually less than that observed before DTT. (iii) Carbachol, superimposed on a BAC-depolarization, produced a reversible increment in depolarization, even when additional BAC itself had no further effect. This suggests that not all of the receptors were permanently affected by 1 mM DTT.

(c) Hexamethonium—Hexamethonium retained its normal antagonistic action (as is apparent in Figure 1) and showed no evidence of agonist action. We have not yet determined whether its affinity constant is changed.

Discussion

These experiments are essentially qualitative; further quantitation of (e.g.) changes in apparent dissociation constants are clearly called for, together with measurements of conductance changes to obviate secondary effects of the undoubtedly-substantial changes in ionic gradients (cf. Brown & Scholfield, 1974). Nevertheless, certain points of interest emerge.

Firstly, there is a clear similarity between the effects of DTT on the actions of carbachol and BAC on the ganglion and those previously reported for electroplax by Silman & Karlin (1969). The effect on carbachol is compatible with a reduction in affinity constant. With respect to the irreversible effect of BAC, Silman & Karlin (1969) postulated the presence of an S–S group adjacent to the anionic subsite of the receptor with which the quaternary head-group of BAC interacts. After reduction of the S–S bond to free –SH groups, interaction with the anionic subsite facilitates a covalent reaction between the –SH group and the bromo-acetyl moiety, which in turn sustains the interaction of the quaternary head-group with the anionic subsite. Addition of hexamethonium blocks the latter without disrupting the covalent link, so that renewed depolarization occurs on washing out the hexamethonium. It remains unclear why the irreversible reaction should not go to completion in the continued presence of BAC, instead of attaining a concentration-dependent ceiling.

Secondly, a point of difference from both electroplax and chick biventer muscle emerges in that, in the ganglion, hexamethonium showed no clear agonist action after DTT (cf. Karlin & Winnik, 1968; Rang & Ritter, 1971). This suggests a closer resemblance of the ganglionic nicotinic receptor to that for mono-quaternary nicotinic agonists in leech dorsal muscle (Flacke & Yeoh, 1968; Ross & Triggle, 1972). This analogy is strengthened by observations that α-bungarotoxin is ineffective on both carbachol receptors in leech muscle (Ross & Triggle, 1972) and on ganglionic nicotinic receptors (Magazanik, Ivanov & Lukomskaya, 1974; D.A. Brown, unpublished observations), in contrast to its well-known action on electroplax and vertebrate striated muscle.

This study was aided by a grant from the American Medical Association Education & Research Foundation to D.A.B. The authors thank Dr J.F. Collins of the City of London Polytechnic for synthesizing BAC and Professor C.Y. Chiou for a gift of the same.

References

ALBUQUERQUE, E.X., SOKOLL, M.D., SONESSON, B. & THESLEFF, S. (1968). Studies on the nature of the cholinergic receptor. *Eur J. Pharmac*, **4**, 40–46.

BEN-HAIM, D., DREYER, F. & PEPER, K.(1975). Acetylcholine receptor: modification of synaptic gating mechanism after treatment with a disulfide bond reducing agent. *Pflüg. Arch.*, **355**, 19–26.

BEN-HAIM, D, LANDAU, E.M. & SILMAN, I.(1973). The role of a reactive disulphide bond in the function of the acetylcholine receptor at the frog neuromuscular junction. *J. Physiol., Lond.*, **234**, 305–325.

BROWN, D.A., BROWNSTEIN, M.J. & SCHOLFIELD, C.N (1972). Origin of the after-hyperpolarization that follows removal of depolarizing agents from the isolated superior cervical ganglion of the rat. *Br. J. Pharmac.*, **44**, 651–671.

BROWN, D.A. & SCHOLFIELD, C.N.(1974). Changes of intracellular sodium and potassium ion concentrations in isolated rat superior cervical ganglia induced by depolarizing agents. *J. Physiol., Lond.*, **242**, 307–319.

BROWN, D.A. & MARSH, S.(1975). A very simple method for recording ganglion depolarization. *J. Physiol., Lond.*, **246**, 24–26P.

CHIOU, C.Y.(1974). Cholinergic receptor labelling: specific irreversible binding of bromoacetylcholine to cholinergic receptors at neuromuscular junctions. *Eur. J. Pharmac.*, **26**, 268–276.

CHIOU, C Y. & RAMA SASTRY, B.V.(1968). Acetylcholine hydrolysis of halogen-substituted acetylcholines. *Biochem. Pharmac.*, **17**, 805–815.

CLELAND, W.W (1964). Dithiothreitol. A new protective reagent for SH groups. *Biochemistry, N.Y.*, **3**, 480–482.

FLACKE, W. & YEOH, T S (1968). Differentiation of acetylcholine and succinylcholine receptors in leech muscle. *Br. J. Pharmac.*, **33**, 154–161.

KARLIN, A. & BARTELS, E.(1966). Effects of blocking sulphydryl groups and of reducing disulfide bonds on the acetylcholine-activated permeability system of the electroplax. *Biochem. biophys. Acta*, **126**, 525–535.

KARLIN, A. & WINNIK, M.(1968). Reduction and specific alkylation of the receptor for acetylcholine. *Proc. Natn. Acad. Sci. U.S.A.*, **60**, 668–674.

MAGAZANIK, L.G., IVANOV, A. YA & LUKOMSKAYA, N. YA(1974). The effect of snake venom polypeptides on cholinoreceptors in isolated rabbit sympathetic ganglia. [In Russian]. *Neurofiziol.*, **6**, 652–656.

MITTAG, T.W. & TORMOY, A. (1970). Disulfide bonds in nicotinic receptors. *Fedn Proc.*, **29**, 547.

RANG, H.P. & RITTER, J.M.(1971). The effect of disulfide bond reduction on the properties of cholinergic receptors in chick muscle. *Molec. Pharmac.*, **7**, 620–631.

ROSS, D.H. & TRIGGLE, D.J.(1972). Further differentiation of cholinergic receptors in leech muscle. *Biochem. Pharmac.*, **21**, 2533–2536.

SILMAN, I. & KARLIN, A.(1969). Acetylcholine receptor: covalent attachment of depolarizing groups at the active site. *Science, N.Y.*, **164**, 1420–1421.

(Received October 10, 1975)

INSTRUCTIONS TO AUTHORS

Papers will be considered for publication on all aspects of drug action, including chemotherapy. A recent issue of the Journal is a good guide to style.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh, EH8 9JZ, from whom copies of the full Instructions to Authors, published in *Br. J. Pharmac,* **50**, 3-23, may also be obtained. Specific points to note are:

1. **Manuscript** should be typed in double spacing on one side of paper not larger than A4 (206 x 294 mm). There should be a separate title page giving the names and addresses of the authors **in alphabetical order.** A short title of not more than 50 letters should also be suggested.

2. **Statement** Papers are accepted only if accompanied by a statement that they have not been and will not be published in whole or in part in any other Journal.

3. **Summary** (2 copies) This should be a short summary of results and conclusions arranged in numbered paragraphs.

4. **Abstract** (2 copies) An abstract of about 100 words suitable for inclusion in an abstracting journal should be typed on a separate sheet, giving the author's name and address and the title of the paper.

5. **Figures** These should not be larger than A4 (206 x 294 mm) and may be in the form of original drawings, recorded tracings or high quality photographic prints made from them. Negative prints of kymograph tracings (black on white) should be provided, and both ordinates and abscissae should be calibrated. Lettering on figures should be inserted in pencil. Symbols which are to appear in figures or legends should be chosen from the following:

○ ● □ ■ △ ▲ ▽ ▼

Photographs and photomicrographs should be printed on glossy paper and should be larger than the size required for reproduction.

6. **Tables** These should not have more than 85 characters to a line (counting spaces between columns as 4 characters) and certainly not more than 110 characters to a line, unless absolutely unavoidable. **Figure legends** and **tables** should be typed on separate sheets of paper.

7. **References** These should be collected in alphabetical order at the end of the paper. They should include the authors' names, year of publication (in brackets), title of article, title of publication (abbreviated in accordance with the fourth edition of the *World List of Scientific Periodicals*), volume number and first and last page numbers. References to books should in addition, include the names of editors, the edition number, where appropriate, and the town of origin and name of publisher.

Two page proofs will be supplied, one of which may be retained by the authors. The other should be corrected immediately and returned to the Press Editor. Corrections should be kept to a minimum.

Twenty-five reprints will be supplied to the authors free of charge. Additional reprints can be purchased. A reprint order form, which should be returned promptly, will be sent out with the proofs. No reprints of abstracts are supplied.

Short Communications These may be accepted if they merit priority publication and do not require revision. They may contain up to 1,200 words of text and only one figure or table. The summary should be a single paragraph. An abstract and statement should be included.

British Journal of Pharmacology

VOLUME 56 NUMBER 1 JANUARY 1976

3 SCHILD, H.O. Dale and the development of pharmacology

9 HILL, R.G., SIMMONDS, M.A. & STRAUGHAN, D.W. Antagonism of γ-aminobutyric acid and glycine by convulsants in the cuneate nucleus of cat

21 GANGULY, D.K. Antioxotremorine action of propranolol

25 BOSIN, T.R., HIXSON, E. JANE & MAICKEL, R.P. Pressor effects of tryptamine analogues

29 OSBORNE, N.N. & PENTREATH, V.W. Effects of 5,7-dihydroxytryptamine on an identified 5-hydroxytryptamine-containing neurone in the central nervous system of the snail *Helix pomatia*

39 GRYGLEWSKI, R.J. & KORBUT, R. Bioassay of histamine in the presence of prostaglandins

43 ADAMS, M.D., CHAIT, L.D. & EARNHARDT, J.T. Tolerance to the cardiovascular effects of Δ^9-tetrahydrocannabinol in the rat

49 GILBERT, J.C. & WYLLIE, M.G. Effects of anticonvulsant and convulsant drugs on the ATPase activities of synaptosomes and their components

59 JOHNSON, A.M., LOEW, D.M. & VIGOURET, J.M. Stimulant properties of bromocriptine on central dopamine receptors in comparison to apomorphine, (+)-amphetamine and L-DOPA

69 BUCKINGHAM, R.E., HAMILTON, T.C. & MOORE, R.A. Prolonged effects of *p*-chlorophenylalanine on the blood pressure of conscious normotensive and DOCA/saline hypertensive rats

77 MILSON, J.A. & PYCOCK, C.J. Effects of drugs acting on cerebral 5-hydroxytryptamine mechanisms on dopamine-dependent turning behaviour in mice

87 JOHNSON, F.N. The effect of lithium chloride on one-trial passive avoidance learning in rats

93 BARBER, H.E., CALVEY, T.N., MUIR, K.T. & TAYLOR, K. The effect of edrophonium on erythrocyte acetylcholinesterase and neuromuscular function in the rat

101 STONE, T.W. Responses of neurones in the cerebral cortex and caudate nucleus to amantadine, amphetamine and dopamine

111 FELTZ, ANNE & LARGE, W.A. Effect of atropine on the decay of miniature end-plate currents at the frog neuromuscular junction

115 DANILOV, A.F., IVANOV, YU.J. & MICHELSON, M.J. The influence of motor nerve tetanization on the effect of organophosphorus cholinesterase inhibitors on neuromuscular transmission

SHORT COMMUNICATIONS

125 BAKHLE, Y.S. & YOUDIM, M.B.H. Metabolism of phenylethylamine in rat isolated perfused lung: evidence for monoamine oxidase 'type B' in lung

128 BROWN, D.A. & KWIATKOWSKI, D. A note on the effect of dithiothreitol (DTT) on the depolarization of isolated sympathetic ganglia by carbachol and bromo-acetylcholine

Printed in Great Britain by The Whitefriars Press Ltd, London and Tonbridge

CURRENT ISSUE

British Journal of Pharmacology

February 1976

12/5

NOT TO BE LENT OUT

Edited for the British Pharmacological Society by

A.W. Cuthbert *(Chairman)* H.M. Adam *(Secretary)*
Margaret Day *(Press Editor)*
J.D. Stephenson *(Assistant Press Editor)*

A.M. Barrett
W.G. Bisset
T.B. Bolton
D.A. Brown
B.A. Callingham
T.J. Crow
F. de Matteis
C.T. Dollery
V. Eisen
E.W. Gill
A.F. Green
D.F. Hawkins
Margarethe Holzbauer
D.B. Hope
J.S. Kelly
G.M. Lees
G.P. Lewis
I.H.M. Main
E.K. Matthews
J.F. Mitchell
P.A. Nasmyth
M.W. Parkes
M. Sandler
A.P. Silverman
M.A. Simmonds
F.M. Sullivan
A. Ungar
R.A. Webster

Papers will be considered for publication on all aspects of drug action, including chemotherapy.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh EH8 9JZ. Authors should consult the Instruction inside the back cover.

The British Journal of Pharmacology is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633. ISSN 0007-1188.

Annual subscription £36.00 ($90 USA and Canada). (Orders must be accompanied by remittance. Prices applicable only to orders received before 31st December 1976.)

Cheques should be made payable to Macmillan Journals Limited: Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

Enquiries concerning advertising space and rates should be addressed to: Advertisement Department, Macmillan Journals Limited, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633.

Copyright © 1976 by the *British Journal of Pharmacology.* All rights of reproduction are reserved in respect of all papers, articles, illustrations, etc., published in this journal in all countries of the world.

Second class postage paid at New York, N.Y.
US Mailing Agent, Air and Sea Freight Inc., 527 Madison Avenue, New York, NY 10022.

CHARLES C THOMAS • PUBLISHER

CELLULAR PHARMACOLOGY OF EXCITABLE TISSUES *edited by* **Toshio Narahashi**, *Duke Univ. Medical Center, Durham, North Carolina. (12 Contributors)* Recent accomplishments in the field of cellular pharmacology are summarized in this multi-authored volume. In-depth discussion is given on the mechanisms of action of various drugs and chemicals on each excitable tissue including the central nervous system, ganglia, nerve fibers, skeletal, smooth and cardiac muscles. References are quoted in each chapter of this text. *'75, 552 pp., 120 il., 15 tables, $35.50*

PULMONARY THROMBOEMBOLISM *edited by* **Kazi Mobin-Uddin**, *Univ. of Kentucky College of Medicine, Lexington, Kentucky. Foreword by* **Ward O. Griffin, Jr.** *(43 Contributors)* In this volume leading experts in the field of thromboembolism present recent advances in the diagnosis, prevention and management of thromboembolic disease which should be of benefit to medical students, practicing physicians and surgeons. Newer concepts in management with anticoagulants and fibrinolytic agents are discussed along with prevention by the use of low dose heparin, platelet suppressive drugs, transvenous caval interruption and the umbrella filter. *'75, 412 pp. (6 3/4 x 9 3/4), 130 il. (6 in full color), 62 tables, $40.00*

TREATMENT OF PAIN *edited by* **Harold C. Voris** *and* **Walter W. Whisler**, *both of Rush Medical College, Chicago, Illinois. (19 Contributors)* An account of the history, knowledge and theories of brain transmission is presented along with studies providing anatomical proof of the complexity of pain pathways and their connections at various levels of the nervous system. Discussed are the use of hypnosis or trance therapy, acupuncture, and new techniques of electrical stimulation to relieve pain. Especially outstanding are the chapters on dorsal or sensory root rhizotomy and sympathectomy. *'75, 176 pp., 28 il., 18 tables, $14.00*

CHEMOTHERAPY OF UROGENITAL TUMORS *by* **Gerald P. Murphy** *and* **Arnold Mittelman**, *Roswell Park Memorial Institute, Buffalo, New York. Foreword by* **Willet F. Whitmore, Jr.** *(4 Contributors)* Current chemotherapy of urogenital neoplasms is critically evaluated in this book. In a multidisciplinary approach to urogenital cancer, the authors carefully examine the clinical and biochemical criteria for judging the effectiveness of chemotherapy. The epidemiologic importance of each neoplasm is reviewed for each disease category. *'75, 288 pp., 15 il., 62 tables, $24.50*

VASOACTIVE SUBSTANCES RELEVANT TO MIGRAINE *edited by* **Seymour Diamond**, *The Chicago Medical School, Chicago, Illinois;* **Donald J. Dalessio**, *Scripps Clinic Medical Group, Inc., La Jolla, California;* **John R. Graham**, *Faulkner Health Care Corporation, Roslindale, Massachusetts; and* **Jose L. Medina**, *The Chicago Medical School, Chicago, Illinois. (15 Contributors)* This text covers the roles of histamine, serotonin, renin, angiotensin, catecholamines, neurokinin, prostaglandins and other substances in the pathogenesis of migraine. The mechanisms of action in the medications used in migraine are described to help physicians understand them more completely. *'75, 112 pp., 24 il., 12 tables, $11.50*

A HANDBOOK ON THE 1970 FEDERAL DRUG ACT: Shifting The Perspective *by* **Robert L. Bogomolny**, *Southern Methodist Univ., Dallas, Texas;* **Michael R. Sonnenreich**, *The National Commission on Marihuana and Dangerous Drugs, Washington, D. C.; and* **Anthony J. Roccograndi**, *Southern Methodist Univ., Dallas, Texas.* This book contains an in-depth analysis of the major revision of drug laws in the United States, including a full description of the federal drug statutes dealing with control of stimulant, depressant, hallucinogenic and narcotic drugs. *'75, 192 pp., 1 il., $13.50*

Orders with remittance sent by air, postpaid

301-327 East Lawrence Avenue • Springfield • Illinois • 62717 • USA

Symposium on Drug Metabolism

6th–8th April 1976

University of Surrey, England

An International Symposium on Drug Metabolism in honour of Professor R. T. Williams, FRS, is to be held at the University of Surrey, England, on 6th-8th April 1976. The programme includes symposia on 'Advances in mechanisms of drug metabolism' and 'Species differences in drug metabolism—from microbe to man'. Sessions will be held for free papers.

Accommodation will be provided on the University campus at Guildford, Surrey, England. Further details including the programme and registration forms may be obtained from Dr. L. J. King, Department of Biochemistry, University of Surrey, Surrey, GU2 5XH.

SPAIN

A leading Spanish pharmaceutical laboratory in Madrid, has a position open for a pharmacologist—preferably Spanish or Spanish speaking—in the Department of Pharmacodynamics of its Research Center. He will be assistant to the head of the Department in the work concerning general and initial pharmacological screening, pharmacological estimation of new drugs.

Applicants must have a minimum experience (postgraduate 5 years) in pharmacological research.

Exclusive dedication. Adequate wage salary. Nice working atmosphere. National and international professional contacts. Applications are absolutely confidential. They must be addressed to

Ref.: Laboratory and Research Department
c/Azcona no. 42
MADRID-28 – SPAIN

British Journal of Pharmacology

VOLUME 56 NUMBER 2 FEBRUARY 1976

133 MARLEY, E. & WHELAN, JENNIFER E. Some pharmacological effects of *p*-chlorophenylalanine unrelated to tryptophan hydroxylase inhibition.

145 BUTTAR, H.S. Impairment in the hepatic clearance of [^{35}S]-bromosulphophthalein in paracetamol-intoxicated rats.

155 BRANDTZAEG, P., GAUTVIK, K.M., NUSTAD, K. & PIERCE, J.V. Rat submandibular gland kallikreins: purification and cellular localization.

169 JURKIEWICZ, A. & JURKIEWICZ, N.H. Dual effect of α-adrenoceptor antagonists in rat isolated vas deferens.

179 ALPS, B.J., DEVOY, P.W. & WATERFALL, J.F. The evaluation of the novel pressor activity of γ-piperidinobutyramide (Wy 20051, DF480).

187 SAXENA, P.N. Sodium and calcium ions in the control of temperature set-point in the pigeon.

193 EVANS, JANE P.M., GRAHAME-SMITH, D.G., GREEN, A.R. & TORDOFF, ANN F.C. Electroconvulsive shock increases the behavioural responses of rats to brain 5-hydroxytryptamine accumulation and central nervous system stimulant drugs.

201 SEIDLER, F.J. & SLOTKIN, T.A. Effects of chronic nicotine administration on the denervated rat adrenal medulla.

209 MICHAL, F. & MOTAMED, MINA. Shape change and aggregation of blood platelets: interaction between the effects of adenosine diphosphate, 5-hydroxytryptamine and adrenaline.

219 ABAITEY, A.K. & PARRATT, J.R. Cardiovascular effects of diethylcarbamazine citrate.

229 FLOWER, R.J., HARVEY, E.A. & KINGSTON, W.P. Inflammatory effects of prostaglandin D_2 in rat and human skin.

235 BUCKINGHAM, JULIA C. & HODGES, J.R. Hypothalamo-pituitary adrenocortical function in the rat after treatment with betamethasone.

Br. J. Pharmac. (1976), **56**, 133–144

SOME PHARMACOLOGICAL EFFECTS OF *p*-CHLOROPHENYLALANINE UNRELATED TO TRYPTOPHAN HYDROXYLASE INHIBITION

E. MARLEY & JENNIFER E. WHELAN[1]
Department of Pharmacology, Institute of Psychiatry, De Crespigny Park, London SE5 8AF

1 Experiments were performed on a variety of tissues from different species to establish whether or not the properties of *p*-chlorophenylalanine methyl ester (PCPA) included a 5-hydroxytryptamine (5-HT)-like action which might explain the soporific action of PCPA in chicks.

2 PCPA, like 5-HT, contracted the rat fundal preparation (as did PCPA base), and in cats enhanced twitch tension of a lower limb flexor reflex, evoked adrenal medullary secretion and attenuated histamine-induced gastric secretion; the effects on the rat fundal strip and the adrenal medulla were prevented by methysergide.

3 Like 5-HT, PCPA elicited bronchoconstriction of guinea-pig lungs, isolated or *in vivo*; this was not prevented by methysergide but reduced by polyphloretin and by indomethacin. Perfusate collected from the lungs during PCPA-induced bronchoconstriction and applied to superfused isolated tissues contained a substance with prostaglandin-like activity.

4 In contrast, the effect of PCPA on the guinea-pig isolated ileum differed from that of 5-HT, since it relaxed the ileum when contracted by transmural excitation, by acetylcholine, histamine or 5-HT and contracted the ileum on wash-out.

Introduction

Most studies of *p*-chlorophenylalanine (PCPA) are concerned with its effects on the sleep-waking cycle, effects attributed to depletion of brain 5-hydroxytryptamine (5-HT) due to tryptophan hydroxylase inhibition (Koe & Weissman, 1966). For example, in various species acute administration of PCPA produced marked insomnia as construed primarily from electrophysiological data (rats—Mouret, Bobillier & Jouvet, 1967; Torda, 1967; cats— Delorme, Froment & Jouvet, 1966; Koella, Feldstein & Czicman, 1968; Johnson, Funderburk, Ruckart & Ward, 1972, and monkeys—Weitzman, Rapport, McGregor & Jacoby, 1968). Nevertheless, Torda (1967) noted that rats were less behaviourally alert after PCPA and Mouret *et al.* (1967) alluded to a dissociation between behavioural and electrocortical changes with PCPA. The rats often appeared to be behaviourally asleep despite alert electrocortical activity. However, Rechtschaffen, Lovell, Freedman, Whitehead & Aldrich (1973) were unable to induce substantial sleep decrements in rats with PCPA, while in cats (Dement, Henriksen & Ferguson, 1973) chronically treated with PCPA (6 to 37 days) wakefulness was initially increased, but substantial amounts of sleep ultimately returned. Depletion of brain 5-HT was verified in both groups.

In contrast, the immediate effects of PCPA in chicks were induction of sleep lasting up to 2 h and a lowering of body temperature (Marley & Whelan, 1974). Both these effects resembled those of 5-HT infused into the chick hypothalamus (Marley & Whelan, 1975). The intention of the present experiments was to compare the properties of PCPA with those of 5-HT on a variety of different species such as the rat (fundal strip, uterus), guinea-pig (lungs and ileum) and cat (gastric secretion, spinal cord reflex, adrenal medullae). A brief account of this work has been presented to the British Pharmacological Society (Marley & Whelan, 1974).

Methods

In vitro *experiments*

Rat isolated fundal strip In 53 experiments the fundal strip was mounted in an organ bath and in 2, superfused, the bathing fluid being Krebs solution at 37°C gassed with 5% CO_2 in O_2 (Vane, 1957, 1964); drug contact was for 90 s on a 5 min cycle. Responses of this tissue, the rat uterus and colon, and the guinea-pig ileum were registered by an isotonic transducer and a pen-recorder.

---

[1] Present address: Department of Pharmacology, South Parks Road, Oxford.

Guinea-pig isolated ileum The ileum (9 animals) was suspended in Krebs solution at 37°C gassed with 5% CO_2 in O_2. For transmural stimulation (Paton, 1955), supramaximal (approx 30 V) rectangular pulses were used, of 1 ms duration at 0.1 Hz; for tetanization, excitation was for 5 to 15 s at 5 hertz. With a drug cycle of 4 min, contact and wash-out were each for 2 min; when PCPA was added to an ileum already contracted for 2 min, this too remained in contact for 2 min (i.e. a 6 min cycle).

Rat isolated uterus A uterine horn (20 rats), from a rat given stilboestrol (100 µg. i.m.) 24 h earlier, was mounted in an organ bath containing rat uterus Ringer solution at 35°C bubbled with O_2 (Ersparmer, 1954; Harvey & Pennefather, 1962). Drug exposure was for 30 s on a 2 or 3 min cycle.

Guinea-pig isolated lungs and superfused assay tissues The lungs (17 guinea-pigs) were removed, suspended in a chamber and perfused at 2.5 ml/min via the pulmonary artery with Krebs solution at 37°C, gassed with 5% CO_2 in O_2. The cannulated trachea was connected to a modified Konzett & Rossler (1940) apparatus and ventilated with oxygen by a pump (60/min, stroke volume 6–10 ml). In some experiments, a rat fundal strip and colon were mounted in series and superfused at 5 ml/min with Krebs solution containing antagonists (µmol/l: methysergide 0.05; propranolol 0.82; hyoscine 0.34; phenoxybenzamine 0.044; mepyramine 0.035) in concentrations found (Piper & Vane, 1969; 1971) to prevent response to 5-HT, catecholamines, acetylcholine and histamine. When the solution containing antagonists had superfused the tissues for at least 60 min, the lung perfusate was passed over the tissues together with the superfusing fluid (Piper & Vane, 1969). Drugs were injected into the perfusing fluid as it entered the pulmonary artery.

In vivo *experiments*

Guinea-pigs Ten guinea-pigs (0.6–0.8 kg) were anaesthetized with urethane (10 ml/kg i.p. of a 12.5% w/v solution). The trachea was cannulated and the animals artificially ventilated, spontaneous respiration being suppressed by injection of urethane (3% w/v) intravenously. Resistance of the lungs to inflation was recorded by a modification of the Konzett & Rossler (1940) method, in which the movements of the piston-recorder were relayed to an isotonic transducer. Injections were given via a jugular cannula.

Chickens A jugular cannula, thermistor, electrocortical and electromyographic electrodes, were implanted in chicks under halothane anaesthesia (Allen, Garg & Marley, 1970). At least 24 h after recovery, behaviour, electrocortical and electromyographic activities were recorded in a temperature-controlled, soundproof box (Marley & Stephenson, 1970). Chicks tested with *p*-chlorophenethylamine were implanted with a jugular cannula only.

Cats Anaesthesia was induced with ethyl chloride and ether, and the trachea cannulated. Except for cats in which the lower limb flexor reflex was being recorded, when anaesthesia was continued with halothane (see below), anaesthesia was maintained by chloralose (80 mg/kg i.v.). Blood pressure was recorded from a carotid or a femoral artery by a blood pressure transducer and pen recorder.

Lower limb flexor reflex Cats were made spinal, by destroying the brain through the approach for the encéphale isolé described by Bradley & Key (1958); the spinal cord was then transected at the junction of the thoracic and lumbar regions and halothane anaesthesia terminated. After fixing the leg by a drill through the lower end of the femur, the reflex was elicited by stimulating the central end of the divided posterior tibial nerve with supramaximal rectangular pulses of 0.5 ms duration at 0.08–0.12 hertz. Responses of the tibialis anterior were transmitted to a Brown-Schuster myograph plate linked to an isotonic transducer and a pen-recorder.

Assay for suprarenal catecholamine secretion Cats (2.5–4.0 kg) which had undergone bilateral lumbar sympathectomy 5–14 days previously, were anaesthetized as above and artificially respired. After abdominal evisceration, the renal vessels were ligated as was the aorta immediately posterior to them. A Gordh needle was tied into the superior mesenteric artery, after retrograde insertion, and heparin (10 mg/kg) injected intra-arterially.

Blood was withdrawn from a carotid artery at 10–15 ml/min through silicone rubber-tubing by a roller-pump (Saxby, Siddiqi & Walker, 1960) driven by a Servomex motor-controller. The blood, warmed in a water-jacket at 40°C, then superfused a rat fundal strip and a chick rectum in series (Vane, 1964); these tissues had been superfused with Krebs solution containing methysergide, 0.1 µM. The blood was then collected in a reservoir and returned to the cat, part by gravity feed to one jugular vein, and part via the roller-pump to the other. The blood volume in the reservoir was kept constant by a photo-sensitive device controlling blood return via the roller-pump (Allen, 1974). 5-HT, PCPA and methysergide were injected directly to the suprarenal glands via the Gordh needle. Control responses of the tissues were obtained by injection of adrenaline via a cannula in the inferior vena cava with its tip opposite the suprarenal glands.

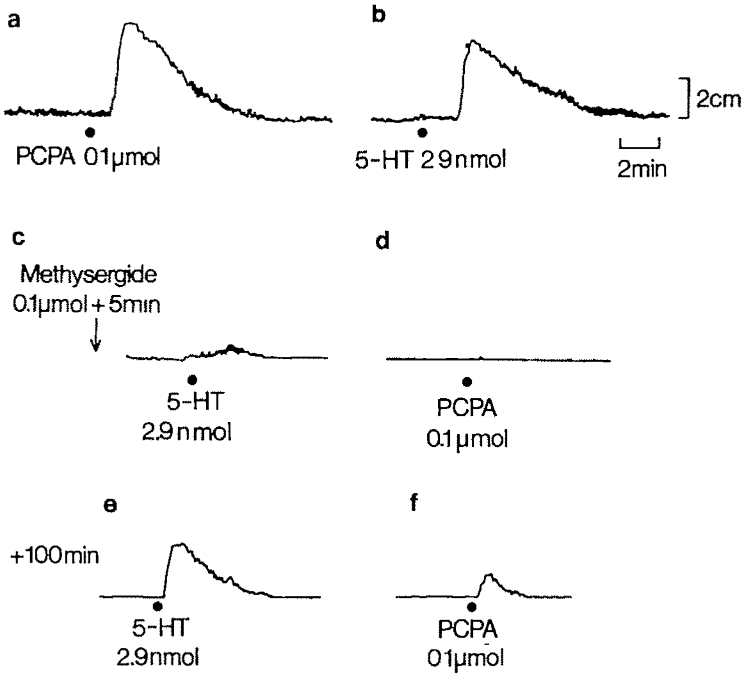


Figure 1 Contractions of the rat fundal strip, superfused with Krebs solution, by *p*-chlorophenylalanine (PCPA) and 5-hydroxytryptamine (5-HT) and their antagonism by methysergide (a) and (b) Contractions of the rat fundal strip by PCPA, 1 µmol, and to 5-HT, 2.9 nmol (c) and (d) Effect of PCPA was abolished, and that of 5-HT substantially reduced, by methysergide, 0.1 µmol. (e) and (f) Partial recovery of responses to 5-HT and PCPA, 100 min after methysergide.

Gastric secretion Three cats (2.5–4.0 kg) were allowed water but deprived of food overnight. The cats were then anaesthetized, the abdomen opened, the pyloroduodenal junction ligated and a cannula (2.5 cm o.d.) tied into the gastric greater curvature. An arterial cannula was passed in the aorta, so that its tip lay immediately anterior to the origin of the coeliac artery. The distal end of the oesophagus was then ligated and the vagi divided in the neck. After washing out the stomach with warm saline, the abdomen was closed round the cannula and the animal laid on its side. Histamine (3.0 nmol kg^{-1}min^{-1}) was then continuously infused via a femoral vein and gastric juice collected in graduated tubes. When the volume of histamine-induced gastric secretion was reasonably constant (4–6 ml/15 min), 5-HT or PCPA was infused via the aortic cannula for 30 minutes. For estimation of free and total HCl, the specimens were titrated with 0.1N NaOH after the addition of Topfers reagent (Bayliss, 1954).

Drugs

The drugs used included the hydrochlorides of β-phenethylamine, phenoxybenzamine, (±)-phenylisopropylhydrazine, (±)-propranolol, Ro4-4602 (N^1-(DL-seryl)-N^2(2,3,4 trihydroxybenzylhydrazine)), and tryptamine. Also used were acetylcholine perchlorate, (−)-hyoscine hydrobromide, 5-hydroxytryptamine creatinine sulphate, indomethacin, (±)-mebanazine oxalate, mepyramine maleate, methysergide bimaleate, *p*-chlorophenethylamine, *p*-chloro-(±)DL-phenylalanine and its methyl ester, L-phenylalanine and its methyl ester, phenelzine hydrogen sulphate and tetrodotoxin. Prostaglandin E$_1$ and prostaglandin F$_{2\alpha}$·tromethamine salt (0.2 mg/ml) were dissolved in ethanol and 0.9% saline containing sodium carbonate, (Bennett & Posner, 1971). Polyphloretin phosphate (100 mg/ml) was dissolved in distilled water and the pH adjusted to 7.0 with sodium carbonate.

Results

Section A. 5-hydroxytryptamine-like effects of p-*chlorophenylalanine*

Rat stomach strip The superfused rat fundal strip which is extremely sensitive to 5-HT (Vane, 1957) was contracted by PCPA in the presence of hyoscine and almost identical contractions could be elicited by

PCPA, 100 nmol and 5-HT, 2.9 nmol (Figures 1a and b). The responses to PCPA and 5-HT were virtually abolished by a single injection of methysergide, 0.1 µmol (Figures 1c and d), and partial recovery occurred 100 min later (Figures 1e and f).

In experiments with the rat fundal strip bathed in a conventional organ bath, PCPA base (0.2, 0.4 and 2.5 µmol) was also found to contract the tissue, and as with the methyl ester, its effects were prevented by methysergide although antagonism was surmounted by increasing the dose of PCPA (base). The possibility that PCPA and 5-HT contracted the fundal strip by reacting with the same receptors was explored further by testing the tissue's response to serial doses of PCPA and of 5-HT, and by examining the interaction of PCPA with β-phenethylamine and dexamphetamine, substances thought to contract the fundal strip by activation of 5-HT receptors (Vane, 1960). The results are illustrated in Figure 2. Indeed, PCPA exhibited cross-tachyphylaxis with 5-HT (Figures 2b, d), with β-phenethylamine (Figures 2e, f) and possibly with dexamphetamine (Figures 2g, h). Whereas serial doses of PCPA led initially merely to brief reduction followed by recovery in amplitude of responses (Figure 2c), continued serial dosage adversely affected the tissue, the magnitude of responses waning until they eventually disappeared, when contractions evoked by 5-HT were difficult to elicit. This decline in effect of PCPA was not due to inability of the muscle to respond, since the response to acetylcholine, 8 pmol, was unaffected. Following even a single 'priming' dose of PCPA, the magnitude of the responses to dexamphetamine became erratic (Figure 2h). The addition of hyoscine (0.1 µM), mepyramine (10 µM) and occasionally propranolol (0.6 µM) to the bathing fluid, did not alter the contractions to 5-HT, PCPA, β-phenethylamine or dexamphetamine.

Since desensitization by PCPA not only affected responses to 5-HT but also to dexamphetamine and β-phenethylamine, dose-response data were obtained to these four substances. The results are given in Table 1. Dose-response curves for 5-HT, β-phenethylamine and PCPA were reasonably parallel, further evidence

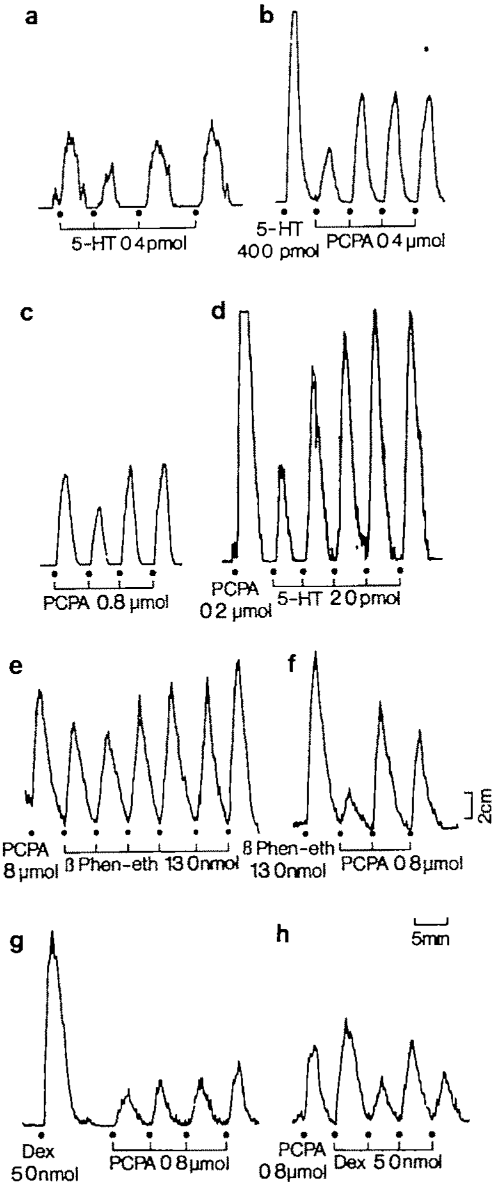


Figure 2 Responses of 8 rat fundal strips (a–h) to 5-hydroxytryptamine (5-HT), *p*-chlorophenylalanine (PCPA), β-phenethylamine (βPhen-eth) and dexamphetamine (Dex) The effect of an initial (priming) dose of one drug was observed on subsequent responses to the same or other drugs. (a) Initial diminution, with subsequent recovery of response to 5-HT, 0.4 µmol, following a priming dose of 5-HT. (The first dose interval was 5 min, but duration of subsequent dose-intervals was increased.) (b) Initial diminution, with subsequent increase of response to PCPA, 0 4 µmol, following a priming dose of 5-HT, 40 pmol (c), (d) and (e). Similar recovery patterns as in (a) or (b), but priming doses of PCPA (0.8 µmol, 0.2 µmol and 0.8 µmol respectively) with subsequent doses of PCPA, 0.8 µmol; 5-HT, 2 pmol, or β-phenethylamine, 13 nmol, respectively). (f) After a priming dose of β-phenethylamine, responses to PCPA, 0.8 µmol, became erratic, erratic responses also to dexamphetamine in (h) following a priming dose of PCPA. (g) Gradual increase in response to PCPA, 0.8 µmol, following a priming dose of dexamphetamine, 5 nmol; interval between dose of dexamphetamine and first dose of PCPA was 10 minutes. Doses in this Figure and Figures 4 and 5 are expressed as the final drug concentration per ml of bath fluid.

of an action mediated via 5-HT receptors, whereas that for dexamphetamine was unexpectedly flatter.

There remains the difficulty of explaining how PCPA, a zwitterion in which the algebraic sum of the electric charges on the basic and acidic substituents on the α-carbon atom of the side-chain should approximate zero at neutral or near neutral pH, could exert spasmogenic effects. Four possibilities were examined.

Effects of pH on the response to p-*chlorophenylalanine* Experiments were performed with two rat fundal strips bathed in Krebs solution over the pH range, 2.0 to 8.5. With solutions of pH 2.0 to 7.0, responses to PCPA, 10 μmol, varied between +8% and −6% from the mean, with peak effects at pH 6.0. Evidence favouring the importance of ionization of the amine group came from results obtained at higher pH values, since response to PCPA declined at pH 8.0 to 66% of the mean and was zero at pH 8.5, a pH at which the carboxyl but not the amino group was likely to be ionized.

Decarboxylation of p-*chlorophenylalanine* A net cationic charge at the amino group would also be achieved if PCPA was rapidly decarboxylated to one of its metabolites, *p*-chloro-phenethylamine, a substance which contracts the fundal strip. To exclude this possibility, 6 rats were pretreated with a decarboxylase inhibitor, Ro4-4602 (335 μmol/kg i.p. 4 h previously), to prevent such decarboxylation. However, effects of PCPA (10 μmol) were unimpaired on fundal strips taken from these animals.

Possible release of intraneuronal 5-hydroxytryptamine by p-*chlorophenylalanine* There remained a possibility that PCPA evoked contractions of the rat fundus by release of intraneuronal 5-HT. This possibility was excluded by 2 types of test: (1) Pretreatment with PCPA. Rats were pretreated for 3 days with PCPA, 120 μmol/kg intraperitoneally, a dose that reduces gut 5-HT concentration to less than 30% (Koe & Weissman, 1966). The response of the fundal strip to 5-HT or PCPA was scarcely altered. (2) Monoamine oxidase inhibition. If contraction of the rat fundal strip by PCPA was mediated by the release of intraneuronal 5-HT, the response to PCPA but not that to exogenous 5-HT would be potentiated by a monoamine oxidase inhibitor. However, exposure of the rat stomach strip to a monoamine oxidase inhibitor, mebanazine (5 nmol/ml) added to the bathing solution, only slightly enhanced responses to 5-HT and PCPA. In contrast, the response to tryptamine was greatly enhanced proving that sufficient mebanazine was present to bring about inhibition of the monoamine oxidase. Similar results were obtained with two other monoamine oxidase inhibitors, phenylisopropylhydrazine (3 nmol/ml) and phenelzine (3 nmol/ml). Consequently, the likelihood is that the contractor effects of PCPA were due to its 5-HT-like properties.

Rat uterus Whereas the rat uterus was extremely sensitive to 5-HT and contracted to doses of 1 nmol or less added to a 10 ml bath, addition of PCPA up to a maximum of 2 μmol only rarely contracted a spontaneously active uterus, but neither relaxed nor contracted uterine horns which showed no spontaneous activity.

Secretion of adrenaline by the cat adrenal medulla In cats, the close arterial injection of 5-HT to the adrenal medulla caused secretion of assayable quantities of catecholamines (Reid, 1952). The present experiments were undertaken in 5 cats with chronically denervated adrenal glands, anaesthetized with chloralose and with blood superfused assay tissues in an extracorporeal circuit. Adrenaline, 1.5 and 3 nmol, injected into the inferior vena cava (hereafter, intravenous), relaxed the blood-superfused chick rectum but had no effect on the rat fundal strip; intravenous noradrenaline 5 and 10 nmol was ineffective. Following 5-HT, 0.06 μmol, and later, PCPA 2 μmol, injected to the adrenal glands via the superior mesenteric artery (hereafter, intra-arterial), the chick rectum relaxed as after adrenaline and the rat fundal strip contracted to 5-HT but did not respond to PCPA. The intra-arterial injections of 5-HT and PCPA elevated the cats' blood pressure. Following intra-arterial methysergide (0.213 μmol) the chick rectum no longer relaxed to 5-HT, 0.06 μmol, or PCPA, 2 μmol, given by the same route even though

Table 1 Analysis of dose-response data

Drug	n	*Dose-ranges*	*Gradient*	*s.d.*
5-hydroxytryptamine	6	*0.29 – 5.8 nmol*	*2.9*	±0.06
p-Chlorophenylalanine	4	*1.0 –10.0* μmol	*2.18*	±0.09
Dexamphetamine	6	0.005– 0.542 μmol	*1.03*	±0.05
β-Phenethylamine	5	0.005– 0.588 μmol	*2.36*	±0.09

* Using a 4 × 4 latin square, giving submaximal doses in random order at 20 min intervals. Each experiment was with a fresh rat fundal strip; *n* = number of experiments.

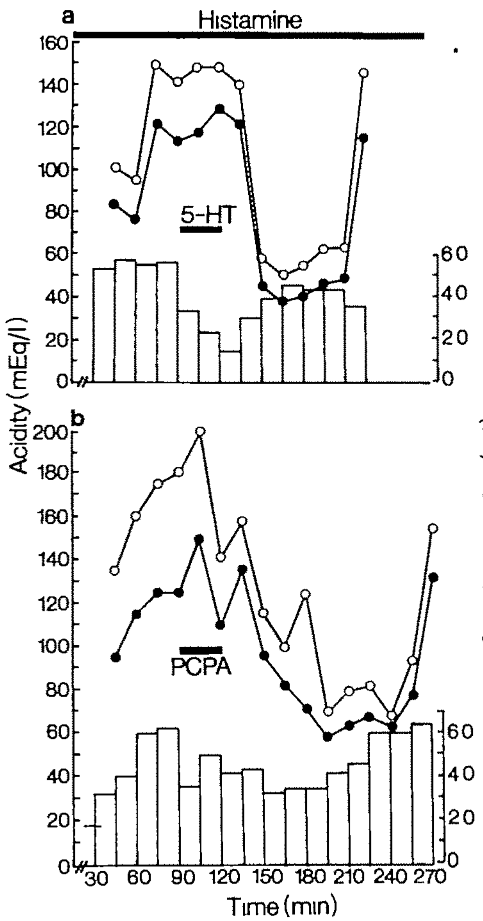


Figure 3 Histograms of gastric secretion and graphs of free and total acidity induced by histamine infused intravenously (3 nmol kg^{-1} min^{-1}) in two cats (a, b) anaesthetized with chloralose. Duration of infusion indicated by black bar. Gastric secretion is expressed as ml on the right of each graph; free acidity (●) and total acidity (○) are expressed as mEq/l HCl on the left. Reduction of free and total acidity, with subsequent recovery, following infusion for 30 min into the aorta (see methods section) of 5-hydroxytryptamine (5-HT), 2.6 nmol kg^{-1} min^{-1} in one cat and of *p*-chlorophenylalanine (PCPA), 0.6 µmol kg^{-1} min^{-1} in another.

relaxation of the chick rectum by intravenous adrenaline was unaltered.

Gastric secretion In 3 cats anaesthetized with chloralose the increase in total and free acidity of gastric secretion evoked by histamine was greatly attenuated following infusion of either 5-HT (2.6 nmol kg^{-1}min^{-1}) or PCPA (0.6 µmol kg^{-1}min^{-1}) (Figures 3a, b), and the volume of gastric juice also diminished.

Spinal cord flexor reflex. In the 4 spinal cats tested, 5-HT and PCPA increased twitch tension of the reflexly excited anterior tibialis muscle. The effects of both substances developed immediately but 5-HT was considerably more potent than PCPA. 5-HT, 2.75 µmol/kg intravenously elevated basal and peak tensions for 10 min whereas a similar elevation of peak tension for 6 min required 240 µmol/kg of PCPA; PCPA did not elevate basal tension. In 2 further cats given strychnine 0.125 µmol/kg, considerable elevation of the peak tension was obtained with as little as 18 µmol/kg of PCPA. 5-HT and PCPA were ineffective after division of the anterior tibial nerve. In contrast, intravenous 5-HT and PCPA in the above doses depressed reflex peak tension in two spinal cats also given chloralose (60 mg/kg i.v.).

In the spinal cats, 5-HT and PCPA elevated blood pressure; in the chloralosed cats they lowered blood pressure before increasing it. These phenomena were probably mediated by different mechanisms, since in the chloralosed cats following division of the vagi in the neck, 5-HT became pressor whereas the effect of PCPA was unaltered.

Behavioural, electrocortical and electromyographic effects in chicks Thirty-one 10–16-day chicks at an ambient temperature of 24°C were given PCPA (8, 10 or 12 µmol/100 g i.v. or i.p.). Behavioural and electrocortical sleep developed within 1 to 2 min, and lasted 1 to 2 h, with disappearance of electromyographic activity from the dorsal neck muscles. Brief arousal was obtainable with sensory stimuli. The development of sleep could not be attributed to formation *in vivo* of *p*-chlorophenethylamine, a centrally active metabolite of PCPA, since *p*-chlorophenethylamine (10 µmol/100 g i.v.) evoked excitation. This developed within 1 min, lasted 5–10 min and resembled that induced by dexamphetamine; *viz* there was behavioural arousal, vocalization, wing abduction, the normal slope of the spine was altered so that the trunk became horizontal, and the respiratory rate increased up to 140/minute.

Section B. Other actions of p*-chlorophenylalanine*

Guinea-pig ileum As noted by Gaddum & Picarelli (1957), 5-HT contracts the longitudinal muscle of the guinea-pig isolated ileum (Figure 4a). In contrast, PCPA 0.5 mmol caused a decrease in basal tone followed by an after-contraction on wash-out (Figure 4b); basal tone was subsequently increased, and even 20 min after the wash-out, tone was still slightly raised. The height and duration of the wash-out contractions were dose-dependent and were unaffected by the addition to the bath of hyoscine, (10 µM), mepyramine, (0.1 µM) or propranolol (2 µM). The

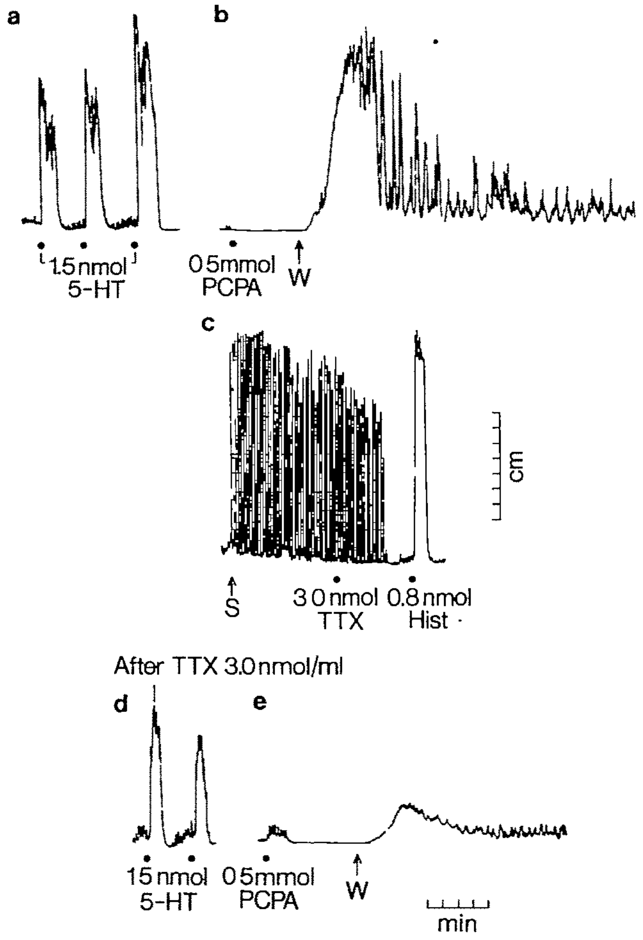


Figure 4 Effects of 5-hydroxytryptamine (5-HT) and *p*-chlorophenylalanine (PCPA) on the longitudinal muscle of a guinea-pig isolated ileum. (a) Contractions to three successive doses of 5-HT, 1 5 nmol. (b) In contrast, PCPA 0.5 mmol slightly reduced resting tone, followed on wash-out of the drug by contraction and increase in basal tone and spontaneous activity. (c) Twitch responses of the electrically excited (S, 0.1 Hz) guinea-pig ileum were abolished by tetrodotoxin (TTX), 3.0 nmol/ml, although the tissue contracted still to histamine (Hist) 0.8 nmol. Following tetrodotoxin, there was (d) diminished response to 5-HT and (e) reduction in amplitude and duration of the wash-out contraction to PCPA

after-contraction to PCPA, 0.5 μmol, (compare Figures 4b, e) and contraction to 5-HT, 1.5 nmol (compare Figures 4a, d) were considerably impaired by the addition of tetrodotoxin (3 nmol/ml) to the bath in sufficient concentration to abolish the response of the tissue to electrical stimulation with coaxial electrodes, yet leave the response to histamine intact.

In addition to decreasing basal tone of the ileum, PCPA, 1 mmol, caused immediate relaxation of the ileum contracted by acetylcholine (6 pmol, Figure 5a; 0.2 nmol, Figure 5f), histamine (0.8 nmol, Figure 5b), 5-HT (3 nmol, Figure 5e), or by coaxial stimulation at 0.1 Hz or 5 Hz (Figures 5c, d).

Bronchoconstriction in guinea-pigs in vivo 5-HT causes bronchoconstriction in intact guinea-pigs (Konzett & Rossler, 1940; Herxheimer, 1953; Konzett, 1956). Sustained bronchoconstriction developed immediately after an intravenous injection of 5-HT, 0.06 μmol; however, PCPA, 6 mmol intravenously caused an immediate bronchoconstriction which first waned over the next 1 to 2 min and then redeveloped even more intensely. Following methysergide (6 μmol i.v.) the bronchoconstrictor effect of 5-HT (0.06 μmol i.v.) was prevented while that of PCPA (6 mmol i.v.) was almost unaffected.

Bronchoconstrictor effects of PCPA (6 mmol i.v.)

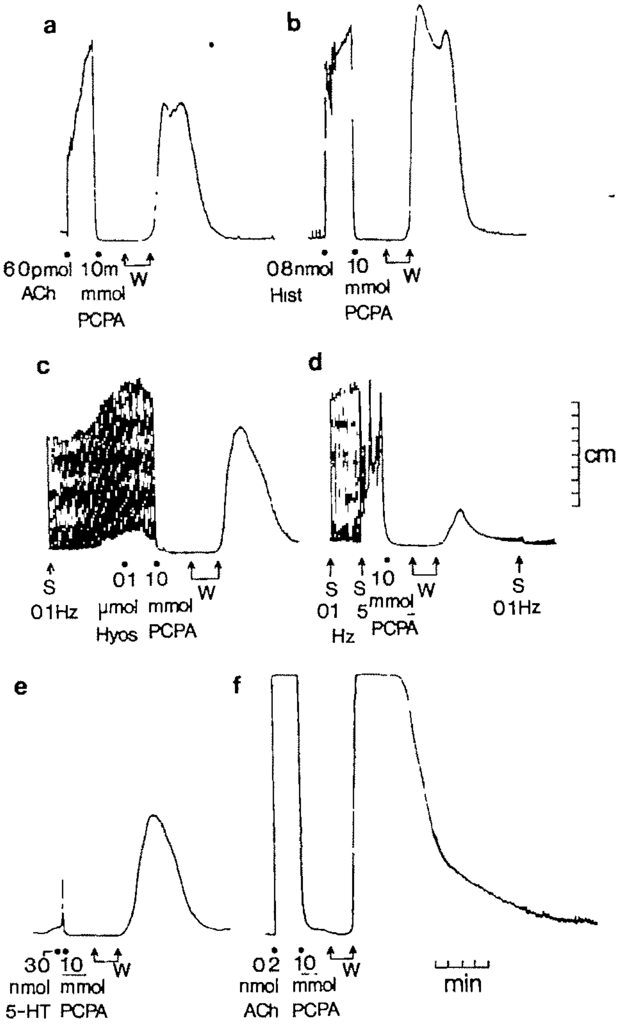


Figure 5 Abolition by *p*-chlorophenylalanine (PCPA), 1 mmol of contractions of different guinea-pig ilea to acetylcholine (ACh), 6 pmol (a) and 0.2 nmol (f); to histamine (Hist), 0.8 nmol (b); to coaxial electrical excitation (S) at 0.1 Hz (c) and 5 Hz (d), and to 5-hydroxytryptamine (5-HT) 3 nmol (e). In (c) Hyos = hyoscine. Wash-out contractions developed in each case.

could not be attributed to histamine release, for whereas mepyramine (25 nmol i.v.) abolished bronchoconstriction due to intravenous histamine (33 nmol), a ten-fold increase in the dose of mepyramine failed to alter the effect of intravenous PCPA (6 mmol). Prostaglandin $F_{2\alpha}$ (13.4 nmol i.v.) mimicked the bronchoconstrictor effects of PCPA. Moreover, the effects of prostaglandin $F_{2\alpha}$ and PCPA were abolished by a prostaglandin antagonist, polyphloretin phosphate (40–45 µg i.v.). In control experiments, intravenous injection of 0.5 ml 70% ethanol, the solvent for prostaglandin $F_{2\alpha}$, lacked effect on bronchial tone.

Bronchoconstriction in guinea-pig isolated lungs 5-HT and PCPA injected into the perfused pulmonary artery induced effects resembling those obtained with intravenous injection *in vivo*. Thus 5-HT, 0.06 µmol and PCPA, 6 mmol induced bronchoconstriction (Figures 6a, b). Addition of methysergide, 6 to 14 µmol/ml, to the perfusate abolished the bronchoconstrictor action of 5-HT (Figure 6c), and slowed the onset of bronchoconstriction due to PCPA, 6 µmol (Figure 6d). Perfusion of the lungs for 30 min with Krebs solution containing indomethacin, 3 nmol/ml, substantially diminished the response to PCPA (6 µmol); bronchoconstriction lasted merely

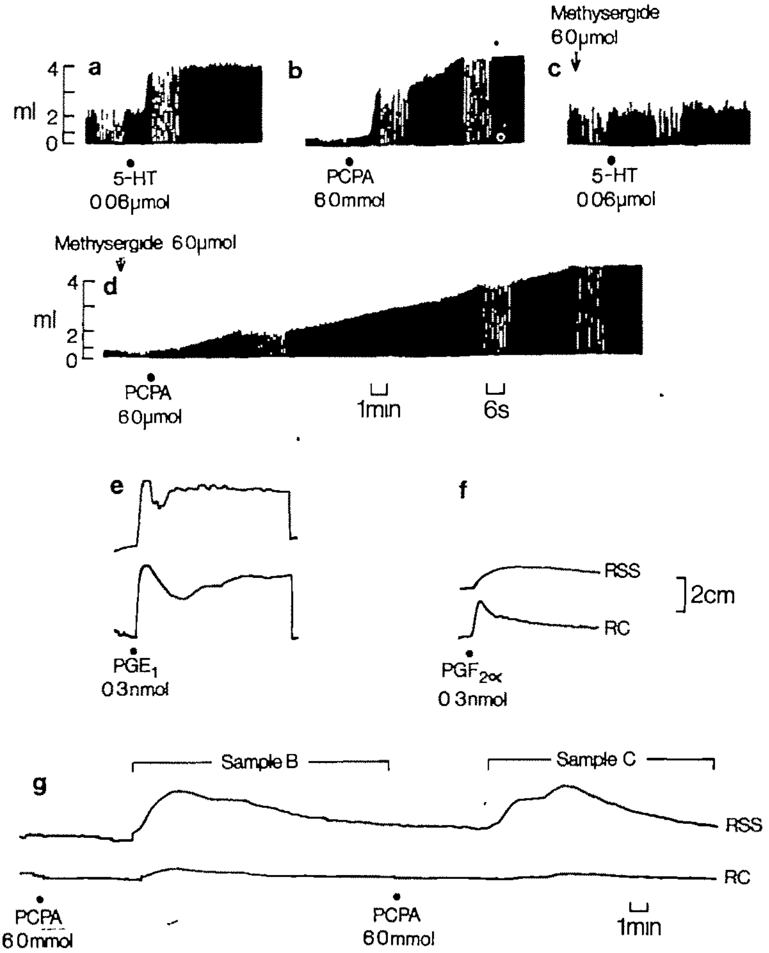


Figure 6 Responses above (a–e) of guinea-pig isolated lungs showing resistance to inflation, and below (e–g) of isolated tissues superfused by perfusate (containing a mixture of antagonists. see methods sections) from another guinea-pig's isolated lungs (RSS = rat fundal strip; RC = rat colon). (a) and (b) Increased resistance to inflation evoked by 5-hydroxytryptamine (5-HT), 0.06 µmol and to *p*-chlorophenylalanine (PCPA), 6 µmol injected into the perfusate entering the pulmonary artery. (c) Effects of 5-HT were abolished by methysergide, 6 µmol, whereas (d) the response to PCPA was slowed. (Responses registered at slow and fast paper speeds.) (e) and (f) Responses of the rat fundal strip and colon to prostaglandins E_1 (PGE_1) and $F_{2\alpha}$ ($PGF_{2\alpha}$), 0.3 nmol, injected directly into the superfusing fluid. (g) Response of the superfused tissues, which developed 5 min after doses of PCPA, 6 mmol, into the perfused pulmonary artery, showing considerable contractions of the rat fundal strip and smaller, simultaneous contractions of the rat colon.

30 s, a finding compatible with PCPA releasing a prostaglandin-like substance capable of evoking bronchoconstriction.

To ascertain whether PCPA released pulmonary prostaglandins, the perfusate from the lungs was superfused over a rat fundal strip and colon (see methods section). These tissues contracted to prostaglandin E_1, 0.07 and 0.3 nmol added to the superfusate, but whereas the rat colon responded to doses of prostaglandin $F_{2\alpha}$ as small as 0.075 nmol, up to 0.3 nmol was required to contract the rat fundal strip (Figures 6e, f).

PCPA (6 mmol) injected into the superfusate passing directly to the rat fundal strip and rat colon had no effect (since methysergide was present), but when injected into the perfusate to the lungs, it increased bronchial resistance and after a delay of 5 min, of which 4 min was required for the perfusate leaving the lungs to reach the superfused tissues, it contracted these (Figure 6g, Sample B). A second dose of PCPA (6 mmol) perfused through the lungs 20 min later, contracted the rat fundal strip but had a smaller effect on the rat colon (Figure 6g, Sample C) and bronchoconstriction was not further intensified.

Discussion

The present research was begun in an attempt to explain the soporific action of PCPA in chicks (Marley & Whelan, 1974), particularly since previous emphasis had been on the 'wakefulness' produced in various other species by this drug. Experiments *in vitro* and *in vivo* revealed at least three hitherto undescribed actions of PCPA.

First, the actions of PCPA *in vitro* on the rat fundal strip and *in vivo* on the cat adrenal medulla, and the antagonism of both by methysergide, are presented as evidence for a direct action of PCPA on 5-HT-like receptors. The effects of PCPA in cats on gastric secretion (see also Fjalland, 1973) and a spinal cord reflex, together with those on behaviour and electrocortical activity in chickens are compatible with 5-HT-like properties (Marley & Whelan, 1974). Evidence that the cationic head of PCPA was crucial for its 5-HT-like activity, derived from experiments using the rat fundal strip. Thus, PCPA still contracted this tissue removed from rats pretreated with Ro4-4602, a substance which inhibits L-aromatic amino acid decarboxylating enzymes. Moreover, PCPA retained its potency on the fundal strip in bathing fluids at acid pH favouring ionization of the amino group, but became ineffective at an alkaline pH at which ionization of the carboxyl group occurs.

PCPA base dissolved in dilute HCl also contracted the tissue, thus excluding the methyl ester moiety as important for the drug's 5-HT-like properties. Release of 5-HT by PCPA was also excluded. The rapid onset of the effects of PCPA and its methyl ester, *in vitro* and *in vivo*, made it unlikely that a metabolite was responsible. Indeed, the only contaminant of PCPA methyl ester revealed by thin-layer chromatography was free PCPA (see Appendix).

Next, whereas 5-HT contracted the guinea-pig ileum there was merely slight relaxation of this tissue to PCPA, although large doses abolished contractions evoked by acetylcholine, histamine, 5-HT or by transmural stimulation. Relaxation of the tissue by PCPA was followed by contraction on wash-out, an effect attenuated by tetrodotoxin, so implicating neuronal as well as smooth muscle components. These phenomena could be explained by a non-specific hyperpolarizing effect of PCPA on the ileal excitable membranes, since the evidence was against interference with calcium influx or an increase in membrane permeability. The different innervation patterns of the guinea-pig ileum and rat stomach would also need consideration in any explanation of these phenomena.

Finally, PCPA elicited bronchoconstriction in guinea-pigs as did 5-HT, but methysergide prevented that to 5-HT but not that to PCPA. However, bronchoconstriction evoked by PCPA was substantially attenuated by indomethacin, and since indomethacin inhibits prostaglandin synthesis (Ferreira, Moncada & Vane, 1971), these results and those of the effect of the lung perfusate on isolated tissues, were compatible with release of a prostaglandin-like substance by PCPA. Although the lungs can fairly readily be provoked into releasing both prostaglandin E_2 and $F_{2\alpha}$ (Piper & Vane, 1971), prostaglandin $F_{2\alpha}$ elicits bronchoconstriction and prostaglandin E_2 bronchodilation (Baum, Wendt, Peters, Butz & Shropshire, 1974), so the fact that PCPA caused bronchoconstriction suggested release of prostaglandin $F_{2\alpha}$, or that release of this substance predominated.

In conclusion, PCPA and its methyl ester possess a number of hitherto undescribed pharmacological properties, in particular a 5-HT-like action and a capacity to release a prostaglandin-like substance from guinea-pig lungs. Although the actual contribution of these effects of PCPA to the overall behavioural status of the animal is beyond the scope of this paper, these should be borne in mind in any evaluation of PCPA activity.

Appendix

Thin layer chromatography on the purity of p-*chlorophenylalanine ester*

These experiments were kindly performed by Sigma Chemical Co. on the sample of PCPA methyl ester used in our experiments. Chromatography was performed using thin-layers of MN-300 cellulose in three solvent systems. The following data were obtained. They indicate that the only contaminant present was free PCPA.

System I. *n*-Butanol : glacial acetic acid : distilled water (60 : 15 : 25)

R_F		R_F	*Markers*
0.96	Major ninhydrin spot	0.96	*p*-chlorophenethylamine
0.89	Minor ninhydrin spot (estimated at 1–2%)	0.89	*p*-chlorophenylalanine
		0.80	phenylalanine
		0.95	phenylalanine methyl ester

System II. Ethyl acetate : pyridine : distilled water : glacial acetic acid (36 : 36 : 21 : 7)

R_F		R_F	*Markers*
0.95	Major ninhydrin spot	0.84	*p*-chlorophenethylamine
0.72	Minor ninhydrin spot (estimated at 1–2%)	0.72	*p*-chlorophenylalanine
		0.61	phenylalanine
		0.92	phenylalanine methyl ester

System III. Methyl ethyl ketone : pyridine : distilled water : glacial acetic acid (70 : 15 : 15 : 2)

R_F		R_F	*Markers*
0.98	Major ninhydrin spot	0.89	*p*-chlorophenethylamine
0.78	Minor ninhydrin spot (estimated at 1–2%)	0.78	*p*-chlorophenylalanine
		0.60	phenylalanine
		0.94	phenylalanine methyl ester

We are indebted to Sigma Chemical Co. for performing the thin-layer chromatography on *p*-chlorophenylalanine and its congeners, and for allowing us to publish their results. We are grateful to the Medical Research Council and to the Bethlem Royal and Maudsley Hospitals Research Fund for financial support. We would like to thank Dr A. Bennett for tetrodotoxin, prostaglandin F$_{2\alpha}$ and polyphloretin; Mercke, Sharp & Dohme for indomethacin, Roche for Ro4-4602 and Sandoz for methysergide. Thanks are due also to Mr D. J. Allen for technical assistance and to Dr A. Brading for helpful discussion on smooth muscle physiology.

This work will form part of J.E.W.'s Ph.D. thesis, University of London. J.E.W. is an M.R.C. scholar.

References

ALLEN, D.J. (1974) A method for regulating return blood flow in an extra-corporeal circuit. *J. Physiol. (Lond.)*, **237**, 17–18P.

ALLEN, D.J., GARG, K.N. & MARLEY, E. (1970). Mode of action of α-methylnoradrenaline on temperature and oxygen consumption in young chickens. *Br. J. Pharmac.*, **38**, 667–687.

BAUM, T., WENDT, R.L., PETERS, J.R., BUTZ, F. & SHROPSHIRE, A.T. (1974). Comparison of activities of two prostaglandin sterioisomers: prostaglandin F$_{2\alpha}$ and prostaglandin F$_{2\beta}$. *Eur. J. Pharmac.*, **25**, 92–100.

BAYLISS, R.I.S. (1954). Technique of determining gastric acidity. In *Practical procedures in clinical medicine: biochemical and radiological investigations*. 2nd ed. p. 31. London: Churchill.

BENNETT, A & POSNER, J. (1971). Studies on prostaglandin antagonists. *Br. J. Pharmac.*, **42**, 584–594.

BRADLEY, P B & KEY, B.J. (1958). The effects of drugs on arousal responses produced by electrical stimulation of the reticular formation of the brain. *Electroenceph. clin. Neurophysiol.*, **10**, 97–110.

DELORME, F., FROMENT, J.L. & JOUVET, M. (1966). Suppression du sommeil par la p.chloraméthamphétamine et la p.chlorophénylalanine. *C. R. Soc. Biol. (Paris)*, **160**, 2347–2351.

DEMENT, W., HENRIKSEN, S. & FERGUSON, J (1973). The effect of the chronic administration of parachlorophenylalanine on sleep parameters in the cat. In *Serotonin and behaviour*, eds Barchas, J. & Ursin, E. pp. 419–424. New York: Academic Press.

ERSPAMER, V. (1954). Pharmacology of indole alkylamines. *Pharmac. Rev.*, **6**, 425–487.

FERREIRA, S.H., MONCADA, S. & VANE, J.R. (1971). Indomethacin and aspirin abolish prostaglandin release from the spleen. *Nature, New Biol.*, **25**, 237–259.

FJALLAND, B (1973). Adrenergic and serotoninergic mechanisms in gastric secretion in rats. *Acta. Pharmac. Toxicol.*, **33**, 103–112.

GADDUM, J.H. & PICARELLI, Z.P. (1957). Two kinds of tryptamine receptor. *Br. J. Pharmac. Chemother.*, **12**, 323–328.

HARVEY, J.A & PENNEFATHER, J.N. (1962). Effect of adrenaline infusions in the catecholamine content of cat and rat tissues. *Br. J. Pharmac.*, **18**, 183–189.

HERXHEIMER, A. (1953). The bronchial reactions of guinea-pigs to 5-hydroxytryptamine. *J. Physiol., Lond.*, **120**, 65P.

JOHNSON, D N., FUNDERBURK, W.H, RUCKART, R T. & WARD, J.W. (1972). Contrasting effects of two 5-hydroxytryptamine-depleting drugs on sleep patterns in cats. *Eur. J. Pharmac.*, **20**, 80–84.

KOE, B.K. & WEISSMAN, A. (1966). Parachlorophenlananine: a special depletor of brain serotonin. *J. Pharmac. exp. Ther.*, **354**, 499–516.

KOELLA, W.P, FELDSTEIN, A. & CZICMAN, J.S (1968). The effects of parachlorophenylalanine on the sleep of cats. *Electroenceph. clin. Neurophysiol.*, **25**, 481–490.

KONZETT, H. (1956). The effects of 5-hydroxytryptamine and its antagonists on tidal air. *Br. J. Pharmac. Chemother.*, **11**, 289P.

KONZETT, H & ROSSLER, R. (1940). Versuchzarordrung zu untersuchungen an der Bronchial resiskulatur. *Arch. exp. Path. Pharmak.*, **195**, 71–74.

MARLEY, E. & STEPHENSON, J.D. (1970). The effects of catecholamines infused into the brain of young chickens. *Br. J. Pharmac.*, **40**, 639–658.

MARLEY, E. & WHELAN, J.E. (1974). Some unexpected pharmacological effects of *p*-chlorophenylalanine methyl ester (PCPA methyl ester). *Br. J. Pharmac.*, **52**, 133–134P.

MARLEY, E. & WHELAN, J.E. (1975). Some central effects of 5-hydroxytryptamine in young chickens at and below thermoneutrality. *Br. J. Pharmac.*, **53**, 37–41.

MOURET, J., BOBILLIER, P. & JOUVET, M. (1967). Effects de la parachlorophénylalanine sur le sommeil du rat. *C. R. Soc. Biol. (Paris)*, **161**, 1500–1603.

PATON, W.D M. (1955). The response of the guinea-pig ileum to electrical stimulation by coaxial electrodes. *J. Physiol., Lond.*, **127**, 40–41P.

PIPER, P.J & VANE, J.R. (1969). Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. *Nature, Lond.*, **223**, 29–35.

PIPER, P.J. & VANE, J.R. (1971). The release of prostaglandins from lung and other tissues. *Ann. N.Y. Acad. Sci.*, **180**, 363–385.

RECHTSCHAFFEN, A., LOVELL, R.A., FREEDMAN, D.X., WHITEHEAD, N.E. & ALDRICH, M. (1973). The effects of parachlorophenylalanine on sleep in the rat; some implications for the 5-hydroxytryptamine sleep hypothesis. In *Serotonin and Behaviour*, eds Barchas, J. & Ursin, E. pp. 401–418. New York: Academic Press.

REID, G. (1952). Circulatory effects of 5-hydroxytryptamine. *J. Physiol., Lond.*, **118**, 435–453.

SAXBY, O.B., SIDDIQI, S. & WALKER, J.M. (1960). Continuous superfusion by means of a simple roller pump. *J. Physiol., Lond.*, **153**, 6–7P.

TORDA, C. (1967). Effect of brain serotonin depletion on sleep in rats. *Brain Res.*, **6**, 375–377.

VANE, J.R. (1957). A sensitive method for the assay of 5-hydroxytryptamine. *Br. J. Pharmac. Chemother.*, **12**, 344–349.

VANE, J.R (1960). The actions of sympathomimetic amines on tryptamine receptors. In *Ciba Foundn Symp. on Adrenergic Mechanisms*, eds Vane, J.R., Wolstenholme, G.E. & O'Connor, M. pp. 356–372. London: Churchill.

VANE, J.R. (1964). The use of isolated organs for detecting active substances in the circulating blood. *Br. J. Pharmac. Chemother.*, **23**, 360–373.

WEITZMAN, E D., RAPPORT, M.M., McGREGOR, P. & JACOBY, J. (1968). Sleep patterns of the monkey and brain serotonin concentrations: effect of parachlorophenylalanine. *Science*, **160**, 1361.

(Received January 13, 1975.
Revised October 2, 1975)

Br. J. Pharmac. (1976), **56**, 145–153

IMPAIRMENT IN THE HEPATIC CLEARANCE OF [^{35}S]-BROMOSULPHOPHTHALEIN IN PARACETAMOL-INTOXICATED RATS

H.S. BUTTAR
Drug Research Laboratories, Health Protection Branch, Health and Welfare Canada,
Ottawa, Canada K1A 0L2

1 The overall functional capacity of the liver was evaluated using [^{35}S]-bromosulphophthalein (BSP, 100 mg/kg, i.v.) in biliary fistulated adult rats pretreated orally with different doses of paracetamol (APAP) for varying time intervals.

2 The maximal hepatic damage occurred between 12–18 h after single doses of APAP (0.5 or 1 g/kg); hepatic excretory function returned to control levels by 48–72 hours.

3 Administration of either 0.5 or 1 g/kg APAP 18 h before BSP caused a dose-dependent inhibition of the choleretic effect of BSP and of the 60 min cumulative excretion of the dye, but conversely, produced a significant increase in the liver and plasma concentrations of ^{35}S.

4 Following acute (0.25 g/kg), or subacute (0.5 g/kg, twice daily for 7 days) treatment with APAP, the total excretion of ^{35}S in bile and the retention of ^{35}S in the liver or plasma remained essentially the same as that for the controls.

5 In rats given single doses of 1 g/kg APAP, the hepatic uptake of the dye was significantly increased during the early stages of intoxication, while the opposite effect was observed at late periods.

6 The bile flow appeared to be inversely related to the excretion of unchanged BSP, and directly related to the excretion of the major BSP conjugate in bile.

7 The hepatic clearance of BSP was more rapid in rats treated subacutely with 0.5 or 1 g/kg APAP, than in those treated acutely with equal doses, suggesting that the intensity of APAP-induced hepatotoxicity became less severe after the repeated administration of this drug.

8 It is concluded that the hepatic uptake, metabolism and excretion of BSP are reversibly impaired following APAP-induced liver injury.

Introduction

Hepatic damage is a well recognized consequence of paracetamol (acetaminophen, N-acetyl-*p*-aminophenol; APAP) over-dosage in humans (Prescott, Wright, Roscoe & Brown, 1971), and in rats (Boyd & Bereczky, 1966; Dixon, Nimmo & Prescott, 1971; Mitchell, Jollow, Potter, Davis, Gillette & Brodie, 1973a). While several biochemical abnormalities indicating hepatocellular damage have been recorded in patients suffering from acute APAP poisoning (Proudfoot & Wright, 1970), information on the overall functional capacity of the liver in the intact organism is lacking. It is generally maintained that the transfer of bromosulphophthalein (BSP) from blood to bile involves three steps: uptake into the liver cells and storage, metabolism, and excretion by the liver. The present study was undertaken to determine which of the hepatic processes concerned with the elimination of BSP from blood to bile would be affected following APAP-induced liver injury.

Methods

Animal treatment

Male Wistar rats (230–280 g) (Woodlyn Farms, Guelph, Ontario) were fed on master laboratory cubes and acclimatized for one week to the laboratory environment before use. They were fasted overnight (12–16 h) before the experiment but had free access to tap water.

Paracetamol was suspended in a 0.25% aqueous solution of gum tragacanth and administered by gavage. The volume of each dose was 2 ml/100 g body weight. Control animals received an equal volume of gum tragacanth vehicle. In the subacute experiment, rats were treated twice daily (09 h 00 min and 20 h 00 min) for 7 days with varying doses of APAP. Control rats received corresponding volumes of the gum tragacanth vehicle over the same period. All rats were weighed daily and received food and water *ad libitum*. The di-sodium salt of [^{35}S]-bromosulphophthalein (BSP), purchased from the Radiochemical Centre, Amersham (sp. act. 33.6 mCi/g), was mixed with unlabelled BSP solution (Hynson, Westcott and Dunning, Inc., Baltimore, Maryland) to give a specific radioactivity of 168 uCi/gram. The radioactive dye was administered as a bolus injection at a dosage of 100 mg/kg body weight.

The common bile duct and femoral vein were cannulated with polyethylene tubing (PX-011) while the rats were under light ether anaesthesia. Since hypothermia is known to reduce bile flow (Roberts, Klaassen & Plaa, 1967), the body temperature was maintained between 37–38°C by means of an electric lamp and the core temperature was monitored with a thermistor inserted about 2.5 cm into the rectum. In order to establish an even biliary flow the first 10 min bile was discarded, then a control bile sample was collected for 30 min before the administration of BSP. Rats were kept in the supine position by light ether anaesthesia throughout the study period.

Collection of body fluids and tissues

Duplicate 10 µl bile and tail blood samples collected at 2, 5, 10, 15, 30, 45 and 60 min after the dye injection were transferred to scintillation vials containing 1 ml of Soluene (Packard Instrument Co., Inc.; Downers Grove, Ill.). Bile was collected for two consecutive 30 min periods. At the end of 1 h, each rat was killed by withdrawing blood from the abdominal aorta in an heparinized syringe, plasma was separated by centrifugation and duplicate 10 µl samples were added to 1 ml of Soluene for measuring the total concentration of BSP. The liver was immediately removed and weighed after blotting off the extraneous blood. Quadruplicate samples (50–100 mg) were taken from different lobes of the liver for estimation of ^{35}S-radioactivity. The bladder was emptied with a syringe, and duplicate 10 µl aliquots of urine were taken for ^{35}S analysis. Bile and urine volumes were measured to the nearest 0.01 ml. The bile samples were kept frozen until analysed for unchanged BSP and metabolites.

Radioactivity determination

Complete solubilization of samples was accomplished by shaking for 24 h at room temperature. The urine and bile samples turned intense blue in the alkaline medium of Soluene. The blue colour was bleached by the addition of 50 µl of glacial acetic acid to the digested samples; it was also added to all other vials including the standards and blanks. Radioactivity was determined with a liquid scintillation counter (Nuclear-Chicago, Isocap-300) after the addition of 15 ml of toluene based scintillation fluid as described earlier (Buttar, Coldwell & Thomas, 1973). Quenching was corrected by the external standard ratios method. The dye concentrations were estimated by computer processing and the values were expressed as unchanged BSP.

Thin layer chromatography of bile

The status of ^{35}S-radioactivity in bile was ascertained by thin layer chromatography (t.l.c.) using Eastman Chromogram sheets (20 x 20 cm No. 6065) impregnated with cellulose and fluorescent indicator and the analytical procedure described by Whelan & Plaa (1963). The two 30 min bile samples were pooled and 2 µl aliquots from various animals were applied to the sheets at a distance of 2 cm apart. Ascending chromatograms were prepared using the upper phase of an organic solvent system consisting of *n*-butanol: glacial acetic acid : water (5 : 1 : 5). The spots were developed by exposing the sheets to concentrated ammonia fumes, air dried, sprayed with 0.25% ninhydrin in ethanol and heated in an oven at 100°C for 3 minutes.

The developed chromatograms were cut into strips (2 cm wide and 16 cm long) and the amount of ^{35}S in each spot was determined by further cutting these strips into sections, each 1 cm wide, and counting each section in a liquid scintillation counter. The ^{35}S radioactivity associated with spots of different R_F-values was expressed as a percentage of the total radioactivity in the bile.

The bile acids (from bile collected for 30 min prior to the dye injection) were extracted, hydrolysed and analysed by gas-liquid chromatography using the method of Watanabe (unpublished). Total tissue water was determined by weighing liver specimens before and after drying to constant weight in an oven at 100°C. The osmolality of bile was checked with an automatic osmometer (Osmette A, Precision Systems Inc.).

Statistics

The statistical significance between test and control groups was determined by Student's *t* test (two-tailed). The difference between means was considered significant when $P < 0.05$. The results were expressed as means with standard errors.

Results

Effects of paracetamol pretreatment on the disappearance of ^{35}S from blood and appearance in bile

When [^{35}S]-BSP was injected intravenously into rats with cannulated bile ducts, ^{35}S was detectable in bile samples taken 2 min after the injection. The highest levels of ^{35}S were observed in bile samples collected at 15 or 30 min intervals from both the control and

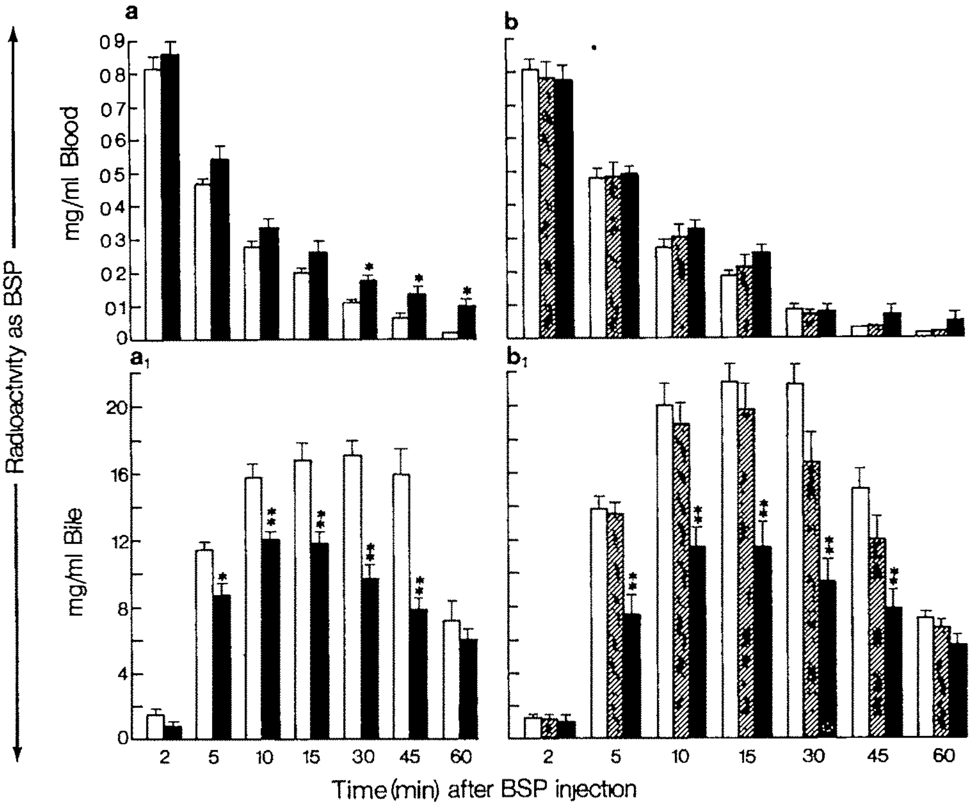


Figure 1 Time course of the disappearance of ^{35}S from the blood and its appearance in the bile during the 60 min period following administration of [^{35}S]-bromosulphophthalein (100 mg/kg, i.v.) (a) and (a$_1$). Rats were pretreated with single doses of paracetamol (1 g/kg, p.o.) and the hepatic function tests were done at 18 h after dosing. Open columns = Controls; solid columns = rats pretreated with paracetamol. (b) and (b$_1$) Rats were pretreated twice daily for 7 days with 0.5 and 1 g/kg paracetamol (p.o.) and the hepatic function tests were done 12 h after the last dose of paracetamol. Control rats received corresponding volumes of 0.25% aqueous gum tragacanth solution. Open columns = controls; cross-hatched columns = paracetamol 0.5 g/kg; solid columns = paracetamol 1.0 g/kg. Each column represents the mean value (± s.e. mean) obtained from 4 to 5 rats. * $P<0.05$; and ** $P<0.01$ when compared with control values.

APAP-poisoned animals, followed by a decline. At the end of 60 min, although the biliary levels of ^{35}S were not significantly different between the control and drug-treated animals, the blood concentrations of ^{35}S were significantly greater in the rats pretreated with APAP (1 g/kg) either 6, 12 or 18 h before the injection of [^{35}S]-BSP. As an example the results obtained 18 h after the acute administration of 1 g/kg APAP are shown in Figure 1a.

When the animals were treated with repeated doses of APAP (1 g/kg) over 7 days, there was again a significant impairment of the excretion of ^{35}S into the bile, but the rate of decline of radioactivity in the blood was similar to that observed in control animals (Figure 1b).

Excretion into bile and retention of ^{35}S in liver of paracetamol pretreated rats

Administration of BSP to control rats resulted in hypersecretion of bile. The choleretic responses were stronger during the 30–60 min than during 0–30 min periods (Table 1). In contrast, significant diminutions in biliary flow were recorded in each of the two 30 min samples following BSP administration to rats treated 6, 12 and 18 h previously with single doses of APAP

(1 g/kg). This effect of APAP persisted up to 24 h, and thereafter the choleretic response to BSP was again observed. The percentage change in biliary flow was calculated as defined by Guarino & Schanker (1968):

$$\frac{\text{ml of bile excreted during 30 min period after BSP injection}}{\text{ml of bile excreted during 30 min period before BSP injection}} \times 100.$$

In rats pretreated for 6, 12 and 18 h before testing the 60 min cumulative recoveries of ^{35}S in bile were 1.5 to 1.9 times smaller, and the quantity of ^{35}S remaining in the liver was 2.9 to 8.5 times greater than the control values (Table 1). These APAP-induced effects disappeared 24 h after dosing.

The urinary excretion of [^{35}S]-BSP-derived radioactivity was negligible both in control (range 0.39–0.48%) and in drug treated animals (range 0.26–1.51%).

Effects of single versus multiple doses of paracetamol on bile flow, biliary and renal excretion of [^{35}S]-bromosulphophthalein and the retention of ^{35}S in the liver

Other tests of liver function such as the activities of serum transaminases (GOT, GPT), the accumulation of triglycerides in liver and histological abnormalities (unpublished observations) had shown that the peak hepatotoxic effects of APAP were induced within 12 to 18 h after dosing; hence 18 h after dosing was chosen as the time at which to study further the effects of APAP on the hepatic clearance of BSP and on its retention in the liver or plasma.

Administration of single doses of 0.25 g/kg APAP did not affect the hepatic clearance of BSP. However, when radioactive dye was injected into rats given single doses of either 0.5 or 1 g/kg of APAP, there was a dose-related inhibition of the choleretic effect of BSP, which was accompanied by a significant decrease in the 60 min cumulative excretion of the dye. At the same time, the retention of ^{35}S in the liver was increased significantly (Table 2).

None of the above mentioned variables was markedly altered in rats receiving 0.5 g/kg APAP for 7 days (Table 2). On the other hand, administration of BSP to rats dosed twice daily for 7 consecutive days with 1 g/kg APAP was followed by a significant reduction both in the biliary flow and in 60 min cumulative excretion of dye, while the retention of ^{35}S in the liver remained essentially unaltered. Rats treated for 7 days showed a 5- to 18-fold greater excretion of [^{35}S]-BSP-derived radioactivity in the urine.

The acute or subacute administration of 0.5 g/kg APAP did not produce any significant changes in the retention of ^{35}S in plasma. However, 60 min after the administration of BSP to rats previously treated with 1 g/kg APAP, the mean plasma concentration of ^{35}S was 5.5 times higher (162.5 ± 27.5 *vs* 29.5 ± 4.1 μg/ml) in the acutely treated rats, and 4 times higher (87.0 ± 15.7 *vs* 21.3 ± 2.3 μg/ml) in subacutely treated rats, than the corresponding control values. Thus, the relatively low levels of ^{35}S in both the liver and plasma of subacutely treated rats suggest that the hepatic clearance of BSP is more rapid in 7 day-treated animals than in animals given single equivalent doses of APAP.

Table 1 Time course of changes in bile flow and in biliary excretion of ^{35}S-radioactivity after the intravenous injection of [^{35}S]-bromosulphophthalein (BSP) (100 mg/kg). Rats were pretreated with single doses of paracetamol (APAP, 1 g/kg, p.o.) and the dye clearance tests were done at different time intervals after dosing

Time BSP injected after dosing with APAP (h)		*Change in bile flow (%)^{c} after injection of BSP*		*^{35}S recovered in bile (% of dose)*			*^{35}S remaining in whole liver after 60 min (% of dose)*
		0–30 min	*30–60 min*	*0–30 min*	*30–60 min*	*Total*	
Controls	(15)^{b}	133.3 ± 2.6^{a}	175.6 ± 6.3	32.8 ± 1.9	41.9 ± 1.8	74.6 ± 1.7	3.5 ± 0.2
6	(5)	62.5 ± 11.6**	78.3 ± 16.6**	22.4 ± 3.2*	24.3 ± 2.6**	46.7 ± 7.5**	30.0 ± 2.7**
12	(4)	54.2 ± 6.8**	64.6 ± 6.4**	18.9 ± 3.3**	22.2 ± 3.9**	41.1 ± 7.0**	24.8 ± 3.2**
18	(5)	88.1 ± 8.1**	112.3 ± 4.8**	20.4 ± 2.7**	21.9 ± 3.1**	42.2 ± 5.6**	10.4 ± 1.6**
24	(4)	168.3 ± 12.1**	125.2 ± 8.8**	51.3 ± 8.7**	19.6 ± 3.6**	70.9 ± 10.0	3.4 ± 0.7
48	(3)	165.9 ± 9.4**	159.4 ± 7.5	53.6 ± 7.7**	22.6 ± 2.4**	76.2 ± 6.1	3.1 ± 0.4
72	(4)	156.3 ± 11.1**	165.5 ± 10.8	51.0 ± 6.9**	27.6 ± 2.9**	78.6 ± 6.7	3.0 ± 0.3

^{a}Values represent the mean ± s.e. mean. ^{b}Figures in parentheses indicate the number of rats in each group. Control data were obtained during each test period, but the variations between the control rats were so small that all the control data were pooled for the final analyses. ^{c}For the calculation of per cent change in bile flow see results section.

* $P < 0.05$; ** $P < 0.01$ when compared with control values.

Table 2 Effect of acute and subacute oral administration of paracetamol (APAP) on bile flow and the biliary and urinary excretion of ^{35}S-radioactivity after the intravenous injection of [^{35}S]-bromosulphophthalein (BSP) (100 mg/kg)

Exptl. condition and dose (g/kg)	*Change in bile flow (%)*[d] *after injection of BSP*		*^{35}S recovered (% of dose)*				*^{35}S remaining in whole liver after 60 min (% of dose)*
	0–30 min	*30–60 min*	*Bile 0–30 min*	*Bile 30–60 min*	*Urine 0–60 min*	*Total*	
Acute[a]							
Control	138.9 ± 7.0[c]	168.3 ± 11.0	38.8 ± 7.4	41.6 ± 4.2	0.36 ± 0.10	80.7 ± 3.9	3.4 ± 0.4
0.25	141.7 ± 4.5	153.6 ± 5.6	46.3 ± 4.2	36.1 ± 2.6	0.36 ± 0.09	82.7 ± 2.2	3.4 ± 0.6
0.50	103.9 ± 3.5*	129.9 ± 8.2*	32.9 ± 4.1	33.6 ± 4.5	0.33 ± 0.06	66.8 ± 5.9*	7.2 ± 1.4*
1.0	88.1 ± 8.1*	112.3 ± 4.2*	20.4 ± 2.7*	21.9 ± 3.1*	0.28 ± 0.08	42.5 ± 5.5*	10.4 ± 1.6*
Subacute[b]							
Control	148.8 ± 5.0	156.4 ± 3.2	48.3 ± 3.7	36.2 ± 2.9	0.22 ± 0.01	84.7 ± 1.9	2.8 ± 0.4
0.50	160.2 ± 10.8	139.0 ± 11.4	56.1 ± 3.2	23.2 ± 4.0	1.06 ± 0.20*	80.6 ± 3.0	3.0 ± 0.4
1.0	120.0 ± 10.5*	114.2 ± 9.3*	34.8 ± 6.9	21.3 ± 3.1*	3.93 ± 1.18*	59.9 ± 6.4*	5.6 ± 1.1

[a] Rats were given single doses of paracetamol (p.o.) and the liver function tests were done 18 h after dosing. [b] Rats were given paracetamol (p.o.) at the dose indicated twice daily for 7 days and the liver function tests were done 12 h after the last dose of paracetamol. Control rats received corresponding volumes of 0.25% aqueous gum tragacanth solution twice daily for 7 days. [c] Values represent the mean ± s.e. mean from groups of 4 to 5 rats. [d] Per cent changes in bile flow were calculated as indicated in the text.
* Indicate those values which are significantly different from the control group at $P < 0.05$.

Time-related effects of paracetamol pretreatment on ^{35}S levels in the liver, bile and plasma

The results summarized in Table 3 show that both liver and plasma concentrations of ^{35}S 60 min after intravenous injection of radioactive dye were significantly greater than control values at 6, 12 and 18 h in rats given 1 g/kg APAP. There was also a decrease in the biliary concentration of ^{35}S and this was statistically significant 18 h after dosing. By 24 h, the liver, bile and plasma concentrations of ^{35}S had returned to control levels.

The bile/plasma concentration ratios of ^{35}S were significantly smaller than their respective control values at 6, 12 and 18 h in 1 g/kg APAP-treated rats, returning to control levels 24 h after dosing (Table 3). The liver/plasma concentration ratio was significantly greater during the first 6 h, while at 18 and 24 h the values of this ratio became significantly smaller than that of the controls, returning to control values by 48 hours.

Effects of paracetamol pretreatment on liver weight, biliary flow rate, bile pH, bile osmolality and bile acids

The liver weight and biliary flow rate were both significantly increased after administering single or multiple doses of 0.5 and 1 g/kg of APAP. The increase in liver weight could not be attributed to the increased amounts of cellular water, because the liver water content remained virtually unchanged in APAP-pretreated rats (Table 4). A significant loss of body weight (not shown) occurred in rats dosed twice daily for 7 days. However, no statistically significant

Table 3 Effect of acute single dose treatments of paracetamol (APAP, 1 g/kg) on liver, bile and plasma levels of ^{35}S-radioactivity 60 min after the intravenous injection of [^{35}S]-bromosulphophthalein (BSP) (100 mg/kg)

Time BSP injected after dosing with APAP (h)		*^{35}S Concentration*			*Concentration ratios*	
		Liver (μg/g)	*Bile (μg/ml)*	*Plasma (μg/ml)*	*Bile/Plasma*	*Liver/Plasma*
Controls	(15)[b]	110.1 ± 7.7[a]	7910.0 ± 318.1	26.4 ± 1 3	303.5 ± 9.9	4 4 ± 0.3
6	(5)	1038.5 ± 77 3**	7262 5 ± 646.4	117.8 ± 19 8**	66.4 ± 11.5**	9.3 ± 1.2**
12	(4)	846.0 ± 90.2**	6700.0 ± 711 3	216 7 ± 33.5**	32.5 ± 3.8**	3.9 ± 0.5
18	(5)	251.3 ± 37.1*	6027.5 ± 437 9*	162 5 ± 27.5**	39 3 ± 4 5**	1 6 ± 0.07**
24	(4)	79.5 ± 13.0	6646.7 ± 698.7	26.3 ± 2.9	268.1 ± 36.2	2.7 ± 0.4*

[a]Values represent the mean ± s.e. mean. [b]Figures in parentheses indicate the number of rats in each group.
* $P<0.05$; ** $P<0.01$ when compared with control values.

Table 4 Effect of acute and subacute administration of various dosages of paracetamol on liver weight and biliary flow rate

Exptl. condition and dose (g/kg)	*Liver wet weight (g/kg body wt.)*	*% liver water content*	*Bile flow rate (ml 30 min^{-1} kg^{-1})*	*Total bile acids (per unit bile vol)*
Acute[a]				
Controls	30.6 ± 1 2[c]	70.3 ± 0.25	1 68 ± 0.04	467 5 ± 35 6
0.25	29.8 ± 0.8	71.0 ± 0.19	2 06 ± 0 16	299.3 ± 19.9*
0.50	39.1 ± 3 1*	70.8 ± 0 20	2 47 ± 0 20*	320.0 ± 37.0*
1.0	41.5 ± 1.2*	72.1 ± 0.22	2 62 ± 0.18*	228.3 ± 66.3**
Subacute[b]				
Controls	31.8 ± 1.6	70.4 ± 0.18	1.67 ± 0.06	530.3 ± 41.9
0.50	41.3 ± 2.6*	71.5 ± 0.20	2.06 ± 0.07*	272.8 ± 36.5**
1.0	44.0 ± 1.9**	71.6 ± 0.24	3.07 ± 0.37*	290.3 ± 46.8*

[a]Rats were given single doses of paracetamol (p.o.) and its effects on various parameters were determined 18 h after dosing. [b]Rats were given paracetamol (p.o.) twice daily for 7 days and the effects on various parameters were determined 12 h after the last dose of paracetamol Control rats received corresponding volumes of aqueous solution of gum tragacanth (0.25%) twice daily for 7 days. [c]Values represent the mean ± s e mean from groups of 4 to 5 rats.
* $P<0.05$, ** $P<0.01$ when compared with control values.

changes in liver weight or biliary flow rate were observed in 7 day-treated rats when calculations were based on the body weight recorded before the initial administration of APAP. The excretion of bile acids was significantly suppressed following the acute or subacute administration of various dosages of APAP, and returned to normal within 48–72 h after administration of the highest dose. No apparent changes were noted either in bile pH (control range 8.09–8.71, APAP-treated range 7.45–8.77), or in bile osmolality (control range 293–321, APAP-treated range 298–331 mOsm/kg H_2O) in the acutely or subacutely treated rats.

Nature of ^{35}S radioactivity in the bile

The t.l.c. of bile showed two major and one minor metabolite of BSP. Standards from radioactive dosing solution of BSP always gave a single peak at a mean R_F-value of 0.74 (range 0.69–0.81). Results in Table 5 show that following the acute administration of 1 g/kg of APAP the biliary excretion of unmetabolized BSP (R_F 0.73) was significantly increased during the first 18 h, returning to control values after 24 hours. In contrast, this treatment significantly reduced excretion of the first major metabolite (R_F 0.33), and correspondingly increased the excretion of

Table 5 Time course of changes in the biliary excretion of unchanged bromosulphophthalein (BSP) and metabolites following the injection of [^{35}S]-BSP (100 mg/kg, i.v.) to paracetamol (APAP, 1 g/kg, p.o) pretreated rats

Time BSP injected after dosing with APAP (h)		*Metabolites*			*Unchanged BSP*
		R_F *0.17 (0.13–0.19)*	R_F *0.33 (0.31–0.38)*	R_F *0.51 (0.50–0.56)*	R_F *0.73 (0.69–0.81)*
Controls	(12)[b]	2.6 ± 0.19[a]	49.6 ± 4.1	27.4 ± 2.3	19.8 ± 1.6
6	(3)	3.8 ± 1.30	30.9 ± 9.6	30.3 ± 7.4	35.1 ± 15.7*
12	(3)	1.5 ± 0.30*	16.0 ± 5.3**	38.3 ± 3.3**	44.3 ± 8.6**
18	(3)	2.2 ± 0.15	22.1 ± 1.9*	46.9 ± 1.8**	28.8 ± 1.3*
24	(4)	1.5 ± 0.40*	42.7 ± 2.5	37.6 ± 1.6	18.9 ± 1.6

[a] The radioactivity associated with different R_F-values (shown as means and ranges) is expressed as a percentage of total ^{35}S present in 1 h bile. Values represent mean ± s.e. mean. [b] Figures in parentheses indicate the number of bile samples analysed.

* $P < 0.05$; ** $P < 0.01$ when compared with control values.

Table 6 Biliary excretion of unchanged bromosulphophthalein (BSP) and metabolites during 1 h following intravenous administration of [^{35}S]-BSP (100 mg/kg) to rats pretreated with different doses of paracetamol

Exptl. condition and dose (g/kg)		*Metabolites*			*Unchanged BSP*
		R_F *0.16 (0.13–0.19)*	R_F *0.35 (0.31–0.38)*	R_F *0.52 (0.50–0.56)*	R_F *0.75 (0.69–0.79)*
Acute[a]					
Controls	(8)[d]	2.2 ± 0.23[c]	50.8 ± 6.5	26.8 ± 3.6	18.4 ± 1.1
0.25	(4)	2.9 ± 0.25	37.2 ± 1.3	39.8 ± 1.4*	20.1 ± 0.9
0.50	(4)	2.9 ± 0.27	33.9 ± 6.3	42.9 ± 5.1*	20.2 ± 1.6
1.0	(3)	2.2 ± 0.15	22.1 ± 1.9**	46.9 ± 1.8*	28.8 ± 1.3**
Subacute[b]					
Controls	(5)	3.1 ± 0.30	62.7 ± 6.6	17.7 ± 4.1	16.4 ± 1.5
0.50	(4)	1.6 ± 0.32*	57.4 ± 1.4	31.4 ± 1.0*	10.0 ± 0.9*
1.0	(4)	0.5 ± 0.01**	42.2 ± 8.9	43.1 ± 6.2*	14.2 ± 4.6

[a] Rats were given single doses of paracetamol (p.o.), and BSP clearance tests were done 18 h after dosing [b] Rats were dosed twice daily for 7 days, and BSP clearance tests were done 12 h after the last dose of paracetamol. Control rats received corresponding volumes of 0.25% aqueous solution of gum tragacanth twice daily for 7 days. [c] The radioactivity associated with different R_F-values (shown as means and ranges) is expressed as a percentage of total ^{35}S-radioactivity present in 1 h bile. Values represent mean ± s.e. mean [d] Figures in parentheses indicate the number of bile samples analysed.

* $P < 0.05$; ** $P < 0.01$ when compared with control values.

the second major metabolite (R_F 0.51) of BSP at 12 and 18 hours. Significantly less of the minor metabolite (R_F 0.17) was detected at 12, 24 and 48 h in acutely dosed rats.

As shown in Table 6, increasing doses of acute APAP caused a corresponding increase in the excretion of the metabolite associated with R_F 0.52, and produced a corresponding decrease in the proportion of the metabolite associated with R_F 0.35. Significantly higher amounts of unchanged BSP were excreted in bile of rats given single doses of 1 g/kg of APAP, as compared with values observed in controls; on the other hand, the excretion of unchanged dye remained similar in gum tragacanth-treated controls and in 7 day APAP (1 g/kg) treated animals. The recovery of unmetabolized BSP was 2-fold smaller in the bile of rats given 0.5 or 1 g/kg of APAP twice daily for 7 days, compared to the amount recovered from the bile of animals given single equivalent doses of this drug. Furthermore, in contrast with the acutely treated rats, the formation of the major BSP metabolite was 1.7-fold and 1.9-fold greater following the subacute administration of 0.5 and 1 g/kg of APAP, respectively (Table 6).

Discussion

The results of this study show that the hepatic disposition of BSP is markedly but reversibly impaired following APAP-induced rat liver damage, and that the hepatotoxic effects depend upon the doses administered. In humans the clinical signs of acute APAP poisoning appear after a latent period of 24 h or more following ingestion and death is usually due to liver failure (Matthew, 1973). A time lag was also noticed in the present study for the full appearance of the hepatotoxic effects of APAP, maximal hepatic damage occurring 12–18 h following the acute administration of this drug, while the hepatic excretory function returning to control levels by 48–72 h (Table 1).

The hepatic clearance of [^{35}S]-BSP-derived radioactivity appeared to be more rapid in rats treated subacutely with APAP, than in those given equal doses on an acute basis (Table 2). Moreover, in contrast with the acutely treated rats, the biliary excretion of unchanged dye was 2-fold smaller, while the production of the major metabolite was 1.7- and 1.9-fold greater after the subacute administration of 0.5 and 1 g/kg of APAP, respectively (Table 6). These observations suggest that the intensity of APAP-induced hepatotoxicity becomes less severe after the repeated administration of this antipyretic/analgesic drug. While the mechanism of adaptation to this hepatotoxin remains unknown, unpublished results with ^{14}C-labelled acetaminophen indicate that the renal elimination of APAP-derived ^{14}C is faster in subacutely treated rats than in their acutely treated counterparts.

The bile to plasma concentration ratio is generally used as a basis for determining the active transport of compounds from plasma to bile. According to Plaa (1971) BSP conjugates are actively transported from plasma to bile. The significantly low bile/plasma concentration ratios observed during 6, 12 and 18 h in animals pretreated with 1 g/kg APAP (Table 3) suggest that the active transfer of BSP and/or its metabolites from liver cells into the bile is impaired following treatment with toxic doses of this drug. Another indicator of overall hepatic excretory function is the liver/plasma BSP concentration ratio. This ratio was significantly high at 6 h, and returned to control levels after 12 h, and at 18 and 24 h the values became significantly smaller in APAP-pretreated rats than that of the controls (Table 3). These results imply that hepatic uptake of the dye is increased during early periods, but is decreased during late periods of APAP intoxication. The reason for this dualistic change in hepatic uptake of BSP in APAP poisoned rats is not immediately clear.

Dyes such as phenol red, fluorescein (Sperber, 1959), and BSP (Klaassen & Plaa, 1968) which are actively transported across the hepatic membrane into the bile generally cause a considerable increase in bile flow due to their osmotic effect. On the other hand, the findings of Priestly & Plaa (1970) suggest that a diminution of bile flow occurs when inhibition of BSP conjugation with glutathione lead to elevated biliary concentration of unconjugated BSP. The results of this study substantiate these previous observations. The administration of BSP to rats treated previously with APAP caused a dose-dependent inhibition of the choleretic effect of BSP (Table 2), and this occurred at a time when increased excretion of unchanged BSP and reduced excretion of the major BSP conjugate were both seen (Tables 5 and 6). It seems unlikely that the temporarily deranged secretion of bile salts would account for the reduced excretion of BSP. Schulze & Czok (1974) have shown that the biliary excretion of BSP remains essentially unchanged in bile salt-depleted and taurocholate-substituted rats.

There is a strong evidence that in rat, man and other mammalian species BSP is mainly conjugated with glutathione (GSH) (Combes, 1964). It has been shown that factors such as the administration of iodomethane (Priestly & Plaa, 1970), or a protein-free diet (Combes, 1965), cause an interference in the hepatic conjugation and subsequently in the biliary excretion of BSP by depleting GSH stores in rat liver. Our unpublished results on the time-related effects of APAP (1 g/kg, p.o.) indicate that the hepatic GSH levels are reduced to a minimum within 3–6 h and restored to normal 24 h after the treatment. Recently Mitchell and coworkers (1973b) have demonstrated that APAP administration causes a dose-dependent depletion of GSH content of mouse liver and pretreatment with cysteine, a GSH precursor, prevents hepatic necrosis. Additional studies have shown that the severity of APAP-induced liver necrosis in various species correlates directly with the rate of hepatic GSH depletion (Davis, Potter, Jollow & Mitchell, 1974). These observations suggest that the reduced formation of the major BSP conjugate and the simultaneously increased excretion of unconjugated BSP found in the present study, are associated with the APAP-caused depletion of hepatic GSH. Other possibilities, such as an APAP-induced defect in the transport of BSP-GSH conjugate from the hepatocytes into the biliary canaliculi, or a non-specific inhibitory action of this drug on the S-aryl glutathione transferase system responsible for BSP conjugation, cannot be excluded.

The results in Table 5 and 6 indicate that the increased biliary excretion of unchanged BSP and the reduced excretion of its major metabolite were always accompanied by an increased excretion of the second major metabolite of this dye. These observations suggest a shift in the primary metabolic pathway of BSP following APAP-induced liver injury. Combes & Stakelum (1960) have suggested that apart from its preferred conjugation with GSH, minor quantities of BSP can be excreted in the form of cysteine, cysteinylglycine and glutamic acid conjugates in rat bile. It may be that when the supply of GSH is reduced, BSP conjugates with the aforementioned amino acids, which are part of the tripeptide glutathione, before its excretion into the bile.

The technical assistance provided by Mr Robert Downie and Miss Isabelle Dupuis is gratefully acknowledged. The author is indebted to Miss H. Watanabe for carrying out the bile acid analyses.

References

BOYD, E.M. & BERECZKY, G.M. (1966). Liver necrosis from paracetamol. *Br. J. Pharmac.*, **26**, 606–614.

BUTTAR, H.S., COLDWELL, B.B. & THOMAS, B.H. (1973). The effect of salicylate on the biliary excretion of ^{14}C-bishydroxycoumarin in rat. *Br. J. Pharmac.*, **48**, 278–287.

COMBES, B. & STAKELUM, G.S. (1960). Conjugation of sulphobromophthalein sodium with glutathione in thioester linkage by the rat. *J. clin. Invest.*, **39**, 1214–1222.

COMBES, B. (1964). Excretory function of the liver. In *The liver morphology, biochemistry, physiology*, ed. Rouiller, C.H., Vol. 2, pp. 1–35. New York: Academic Press.

COMBES, B. (1965). The importance of conjugation with glutathione for sulphobromophthalein sodium (BSP) transfer from blood to bile. *J. clin. Invest.*, **44**, 1214–1224.

DAVIS, D.C., POTTER, W.Z., JOLLOW, D.J. & MITCHELL, J.R. (1974). Species differences in hepatic glutathione depletion, covalent binding and hepatic necrosis after acetaminophen. *Life Sci.*, **14**, 2099–2109.

DIXON, M.F., NIMMO, J. & PRESCOTT, L.F. (1971). Experimental paracetamol-induced hepatic necrosis: A histopathological study. *J. Pathol.*, **103**, 225–229.

GUARINO, A.M. & SCHANKER, L.S. (1968). Biliary excretion of probenecid and its glucuronide. *J. Pharmac. exp. Ther.*, **164**, 387–395.

KLAASSEN, C.D. & PLAA, G.L. (1968). Studies on the mechanism of phenobarbital-enhanced sulphobromophthalein disappearance. *J. Pharmac. exp. Ther.*, **161**, 361–366.

MATTHEW, H. (1973). Acute acetaminophen poisoning. *Clin. Toxicology*, **6**, 9–11.

MITCHELL, J.R., JOLLOW, D.J., POTTER, W.Z., DAVIS, D.C., GILLETTE, J.R. & BRODIE, B.B. (1973a). Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J. Pharmac. exp. Ther.*, **187**, 185–194.

MITCHELL, J.R., JOLLOW, D.J., POTTER, W.Z., GILLETTE, J.R. & BRODIE, B.B. (1973b). Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J. Pharmac. exp. Ther.*, **187**, 211–217.

PLAA, G.L. (1971). Biliary and other routes of excretion of drugs. In *Fundamentals of drug metabolism and drug disposition*, ed. LaDu, B.N., Mandel, H.G. & Way, E.L., pp. 131–145. Baltimore: The Williams and Wilkinson Company.

PRESCOTT, L.F., WRIGHT, N., ROSCOE, P. & BROWN, S.S. (1971). Plasma paracetamol half-life and hepatic necrosis in patients with paracetamol overdosage. *Lancet*, **i**, 519–522.

PRIESTLY, B.G. & PLAA, G.L. (1970). Sulfobromophthalein metabolism and excretion in rats with iodomethane-induced depletion of hepatic glutathione. *J. Pharmac. exp. Ther.*, **174**, 221–231.

PROUDFOOT, A.T. & WRIGHT, N. (1970). Acute paracetamol poisoning. *Br. med. J.*, **3**, 557–558.

ROBERTS, R.J., KLAASSEN, C.D. & PLAA, G.L. (1967). Maximum biliary excretion of bilirubin and sulfobromophthalein during anesthesia-induced alteration of rectal temperature. *Proc. Soc. exp. Biol. Med.*, **125**, 313–316.

SCHULZE, P.J. & CZOK, G. (1974). Studies on the decrease in bile flow produced by sulfobromophthalein. *Toxicol. Appl. Pharmac.*, **28**, 406–417.

SPERBER, I (1959). Secretion of organic anions in the formation of urine and bile. *Pharmac. Rev.*, **11**, 109–134.

WHELAN, F.J. & PLAA, G.L. (1963). The application of thin layer chromatography of sulfobromophthalein metabolism studies. *Toxicol. Appl. Pharmac.*, **5**, 457–463.

(Received March 31, 1975.
Revised August 11, 1975)

Br. J. Pharmac. (1976), **56**, 155–167

RAT SUBMANDIBULAR GLAND KALLIKREINS: PURIFICATION AND CELLULAR LOCALIZATION

P. BRANDTZAEG[1]
Institute of Pathology, University Hospital, Rikshospitalet, Oslo 1
K.M. GAUTVIK
Institute of Physiology, University of Oslo, Oslo 1
K. NUSTAD
Department of Clinical Chemistry, The Norwegian Radium Hospital, Oslo 3 and
Institute for Surgical Research, Rikshospitalet, Oslo 1, Norway
J.V. PIERCE
Hypertension-Endocrine Branch, National Heart and Lung Institute, N.I H.,
Bethesda, Md. 20014, U.S.A.

1 Four submandibular gland kallikreins (E.C. 3.4.21.8) were isolated by chromatography on DEAE-Sephadex A-50 and hydroxyapatite, followed by gel filtration and electrofocusing. The pI values were 3.87, 3.96, 4.07 and 4.16, and a common molecular weight of 34,000 was found.

2 The kallikreins were localized by direct immunofluorescence with an antibody to rat urinary kallikrein, to the granular tubules, striated duct cells and some main duct cells in the submandibular gland, and to striated duct cells in the sublingual gland. Kallikrein was not found in acini and stroma.

3 Several non-kallikrein esterases present in the submandibular gland reacted with the antibody to rat urinary kallikrein. The antibody was made monospecific for kallikrein by absorption with the crossreacting esterases.

4 We suggest that kallikrein is produced in striated duct cells. Granular tubules, which are differentiated from striated duct cells, have preserved the ability to produce kallikrein. These cells also store large quantities of kallikrein.

Introduction

Glandular kallikreins (E.C. 3.4.21.8) are serine proteinases. These enzymes show low affinity for substrates such as casein or haemoglobin, but liberate a biologically active peptide, lysyl-bradykinin (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), from a precursor protein, kininogen, which is found in most biological fluids. Lysyl-bradykinin has pronounced vasodilator effects, and the kallikrein-kinin system may play an important role in the local blood flow regulation in the salivary glands (Hilton, 1970; Gautvik, 1970).

Indirect evidence has suggested that kallikrein is localized in the acini of rat and mouse submandibular glands (Beraldo, Siqueira, Rodriques & Machado, 1972; Bhoola, Dorey & Jones, 1973). We now report the cellular localization of kallikrein in the rat submandibular and sublingual glands using a labelled antibody to rat urinary kallikrein (Nustad & Pierce, 1974).

Methods

Materials

The following substances were obtained commercially: cytochrome C, myoglobin, chymotrypsinogen A, and ovalbumin (Serva-Feinbiochemica, Heidelberg, W. Germany); bovine serum albumin (Behringwerke AG, Marburg, W. Germany); ultrapure sucrose (Mann Research Labs., U.S.A.); DEAE-Sephadex A-50 and Blue Dextran (Pharmacia Fine Chemicals, Uppsala, Sweden); Ampholines (LKB, Bromma, Sweden); acrylamide, N,N'-methylene-bis-acrylamide, ammonium persulphate, N,N,N',N'-tetra methylene diamine and Bio-Gel P-150 (Bio Rad, Labs., U.S.A.); agarose (Litex, Glostrup, Denmark); bradykinin (Sandoz, AG, Basel, Switzerland); tetramethylrhodamine isothiocyanate (Baltimore Biological Laboratory, U.S.A.); trypsin (TRL 2LA, Worthington Biochemical Corp., U.S.A.); yellow casein (nitrated, Calbiochem Inc., U.S.A.); α-

---

[1] Present address: Department of Microbiology, Dental Faculty, University of Oslo, Blindern, Oslo, Norway.

N-benzoyl-L-arginine ethyl ester HCl (Bz-Arg-OEt) and sodium dodecyl sulphate (Sigma Chemical Co., U.S.A.). The dodecyl sulphate was recrystallized after dissolution in absolute alcohol heated to 70°C, filtered and evaporated at room temperature.

Hydroxyapatite was prepared by a new method (Pierce & Nustad, unpublished). Rat urinary kallikrein and the corresponding antiserum were prepared as described by Nustad & Pierce (1974).

Preparation of submandibular gland extract

Twenty female Sprague-Dawley rats (200–300 g) were killed by a blow on the neck. The excised submandibular glands were cut in small pieces and a 30% (w/v) homogenate was prepared in 0.10 M NaCl–0.01M Na-phosphate (pH 7.0) using a Potter-Elvehjem homogenizer (20 strokes, 4°C). The homogenate was centrifuged at 660 × *g* for 5 minutes. The supernatant fluid was treated with Na-desoxycholate (0.5 w/v), dialysed against 0.10 M NaCl–0.01M Na-phosphate (pH 6.0) at 4°C for 30 h, and centrifuged at 20,000 × *g* for 60 minutes. The precipitate was washed and the supernatants combined to give Fraction 1 in 70 ml (Table 1).

Immunoelectrophoresis

Electrophoresis of 2 to 5 µl samples was followed by diffusion against antiserum for 48 h (Laurell, 1966). The plates were pressed, washed and then stained with Coomassie Brilliant Blue (Weeke, 1973).

Immunodiffusion

Micro double diffusion analysis was carried out as described by Brandtzaeg (1970).

Crossed immunoelectrophoresis

Glass plates of 10 × 10 cm were covered with 16 ml of 1% agarose in 0.05 M Tris HCl (pH 8.6) containing 0.2 ml antiserum to rat urinary kallikrein (Nustad & Pierce, 1974). A 2 cm broad strip of solidified agarose was removed along one side and a polyacrylamide gel used for electrofocusing was placed along the cutting edge and fastened with agarose at 50°C without antiserum. Electrophoresis into the antibody-containing agarose was performed with 2 V/cm at 15°C for 16 h with 0.05 M Tris HCl (pH 8.6) in the electrode vessels. The plates were washed, pressed and stained with Coomassie Brilliant Blue or 0.5% Amido Black.

Molecular weight determinations

Rat submandibular esterases were subjected to gel filtration in a Bio-Gel P-150 column at 4°C, with Blue dextran, bovine serum albumin, ovalbumin and myoglobin as standards (Andrews, 1965). Some preparations were also examined by polyacrylamide gel electrophoresis in the presence of dodecyl sulphate. The samples were treated according to Dunker & Rueckert (1969) or by method one of Weber, Pringle & Osborn (1972). Electrophoresis and calculations were carried out as described by Weber *et al.* (1972). Reference proteins were cytochrome C, myoglobin, chymotrypsinogen A, ovalbumin, and bovine serum albumin.

Protein determination

The protein content of main fractions was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin was used as standard (absorbance $A_{280\,nm}^{0.1\%,1\,cm} = 0.64$ in 0.15 M NaCl—0.01 M Na-phosphate, pH 7.4). Protein concentrations in column effluents were measured in a 1 cm cuvette at 280 nm.

Biological assay of kallikrein

Kinin formation was measured on the rat isolated uterus by incubating enzyme fractions with rat kininogen 2, and is expressed as bradykinin equivalents formed per min (Gautvik, Kriz & Lund-Larsen, 1972). The submandibular gland kallikreins contracted the rat isolated uterus when added directly to the organ bath without preincubation with kininogen. This oxytocic activity probably reflects release of kinins from kininogen in the uterus (Nustad & Pierce, 1974), and is specific for the kallikreins (see Results section).

The hypotensive effect of the esterases was measured in rats anaesthetized with Nembutal (40 mg/kg body weight i.p.). Samples of 50 µl were injected into the external jugular vein and the blood pressure recorded through a catheter in the common carotid artery.

Esterase activity

The hydrolysis of α-*N*-benzoyl-L-arginine ethyl-ester (Bz-Arg-OEt) during 5 min at 37°C and at pH 8.5 was determined (Nustad, Pierce & Vaaje, 1975). One esterase unit (EU) was defined as the amount of enzyme that hydrolysed 1 µmole of Bz-Arg-OEt per minute.

Caseinolytic activity

Nitrated casein (yellow casein) 1.5% was incubated with enzyme fractions in 1 ml 0.10 M Na-phosphate (pH 7.4). Samples of 0.4 ml were removed at zero time and after 60 min incubation at 37°C, precipitated with 0.6 ml 1.7 M perchloric acid, stored at 4°C for 1 h, filtered on sintered glass filters (Pyrex G3) and read at 280 nm in a microcuvette (Laake & Vennerød, 1974).

One caseinolytic unit (CU) was defined as the amount of enzyme that increased the A_{280} absorbance of the acidic filtrate by 1 in 1 minute.

Electrofocusing

The method of Nustad & Pierce (1974) was used, except that the pH values of fractions were measured at 4°C rather than at room temperature (Haglund, 1971).

Gel electrofocusing

The method of Wrigley (1968) was used with chemical polymerization and application of the sample on top of the gel, and the anode at the bottom. Gels of 5 × 60 mm or 2.7 × 80 mm were run at 25°C for 4 to 6 h with 200 V constant voltage. The gels were stained and fixed by the method of Malik & Berrie (1972).

Polyacrylamide disc gel electrophoresis, pressure dialysis and phosphate determination

These were carried out as reported by Nustad & Pierce (1974), and conductivity measurements as described by Nustad *et al.* (1975).

Preparation of fluorescent antibody conjugate

A conjugate of the IgG fraction of the sheep antiserum to rat urinary kallikrein B_3 (Nustad & Pierce, 1974) with 35 µg tetramethylrodamine isothiocyanate per mg protein was prepared, filtered through a column of Sephadex G-50, and purified by DEAE-cellulose chromatography (Brandtzaeg, 1973a). The fraction eluted with 0.04 M NaCl–0.01 M Na-phosphate (pH 7.6) was used as the immunofluorescence reagent throughout. Its absorbance ratio A_{280}/A_{515} was 2.1. A working dilution of 0.47 mg/ml was established by performance testing on serial sections of alcohol-fixed rat submandibular glands (see below); it was the lowest conjugate concentration giving duct cell fluorescence of maximum intensity. The fluorescence end point of the conjugate corresponded to 4–8 µg/ml. To abolish non-specific background staining, the conjugate was adsorbed with acetone-extracted mouse liver powder before use, and the sections were preincubated with 25% bovine serum albumin (Brandtzaeg, 1973b).

Immunohistochemical procedures

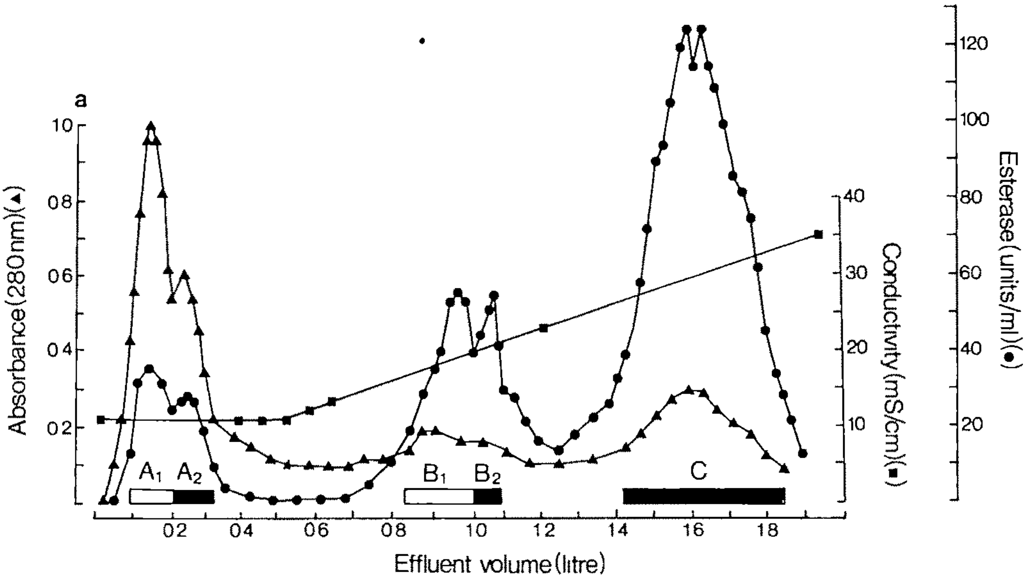
Submandibular and sublingual glands from six adult female Sprague-Dawley rats were collected in ice-cold isotonic saline directly after excision, divided within 20 min into small pieces which were placed in cold 96% ethanol. After further tissue processing, paraffin embedding, and preparation of serial sections (6 µm) (Brandtzaeg, 1974), the sections were incubated with the conjugate for 30 min at room temperature, washed in 0.15 M NaCl–0.01 M Na-phosphate (pH 7.4) and mounted in Evanol (Brandtzaeg, 1973b). Serial control sections were incubated in parallel with aliquots of the conjugate that had been absorbed by addition of esterase B or C (0.17 mg of fraction $2B_1$ and 0.08 mg of 3C per ml working dilution).

Microscopy was carried out with a Leitz Ortholux equipped with an Osram HBO 200 W lamp and a Ploem-type vertical illuminator. Narrow-band excitation and filtration were selected for rhodamine fluorescence (Brandtzaeg, 1973b). The findings were recorded on 'Anscochrome' 500 daylight film.

Results

Purification of submandibular gland kallikreins: esterases C_1–C_4

Rat submandibular kallikreins were purified at 4°C except for step 3 (hydroxyapatite chromatography) which was performed at 20°C (Table 1 and Figure 1). Desoxycholate-solubilized kallikrein (Fraction 1) from homogenates of submandibular glands (see Methods section) was applied in 0.10 M NaCl–0.01 M Na-phosphate (pH 6.0) to a DEAE-Sephadex A-50 column equilibrated with the same buffer. Under these conditions 12% of the total esterase activity was not adsorbed (peak A_1 and A_2). Following application of a linear salt gradient (Figure 1a) 20% of the activity was eluted as a double peak (B_1 and B_2) at a conductivity of 20 mS (0.20 M NaCl–0.01 M Na-phosphate, pH 6.0); 68% of the activity was eluted as an asymmetrical peak (peak C) at 30 mS (0.31 M NaCl–0.01M Na-phosphate, pH 6.0). Peak C was identified as kallikrein by its ability to generate kinins and by immunochemical methods (see below). Peak C was concentrated by pressure dialysis, and equilibrated against 3 M NaCl–0.005 M Na-phosphate (pH 6.0) to give Fraction 2C. Fraction 2C (22 mg) was chromatographed on a hydroxyapatite column (Figure 1b). All of the Bz-Arg-OEt esterase activity was eluted as one peak (3C) with 0.018 M phosphate. Fraction 3C was concentrated by pressure dialysis, equilibrated against 0.01 M Na-phosphate (pH 7.4), and 4.2 mg was filtered through a Bio-Gel P-150 column. Protein and Bz-Arg-OEt esterase activity eluted in the same peak at 1.93 times the void volume (Figure 1c), suggesting a molecular weight of 36,000. A pool was collected, pressure dialysed, and equilibrated against 0.15 M NaCl–0.01 M Na-phosphate (pH 7.4) to give Fraction 4C. This step did not increase the specific activity which was constant throughout the peak (Figure 1c) indicating that hydroxyapatite chromatography produced a highly purified esterase preparation. One ml (4.2 mg) of Fraction 3C was placed in the middle of the stepwise gradient of and electrofocusing column which



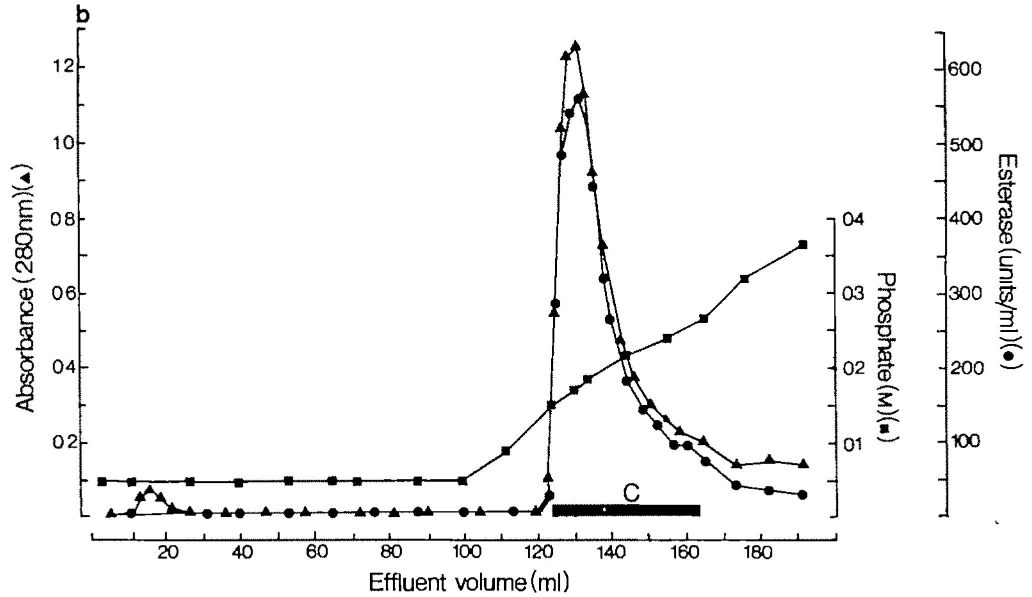
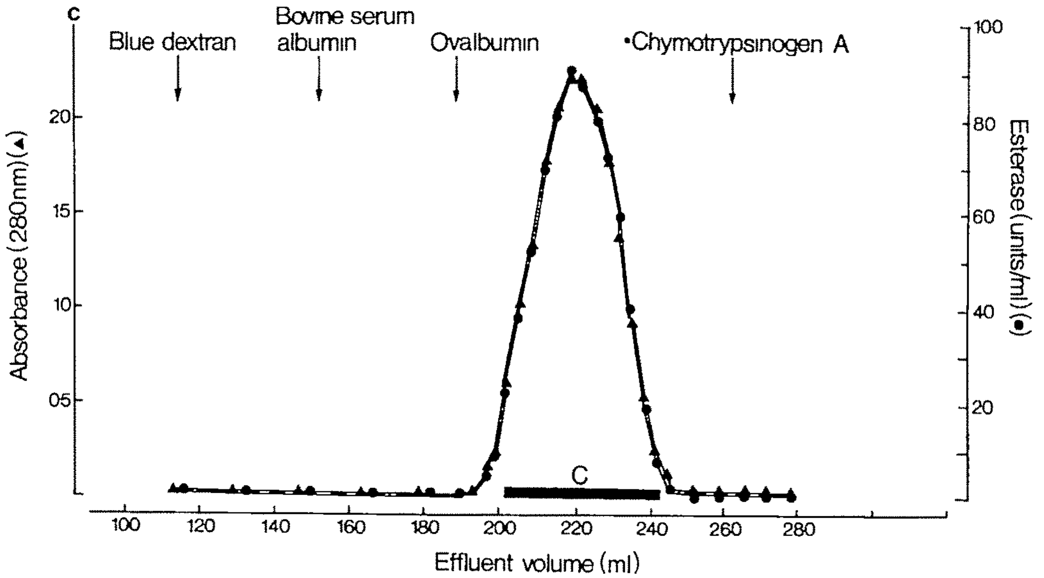
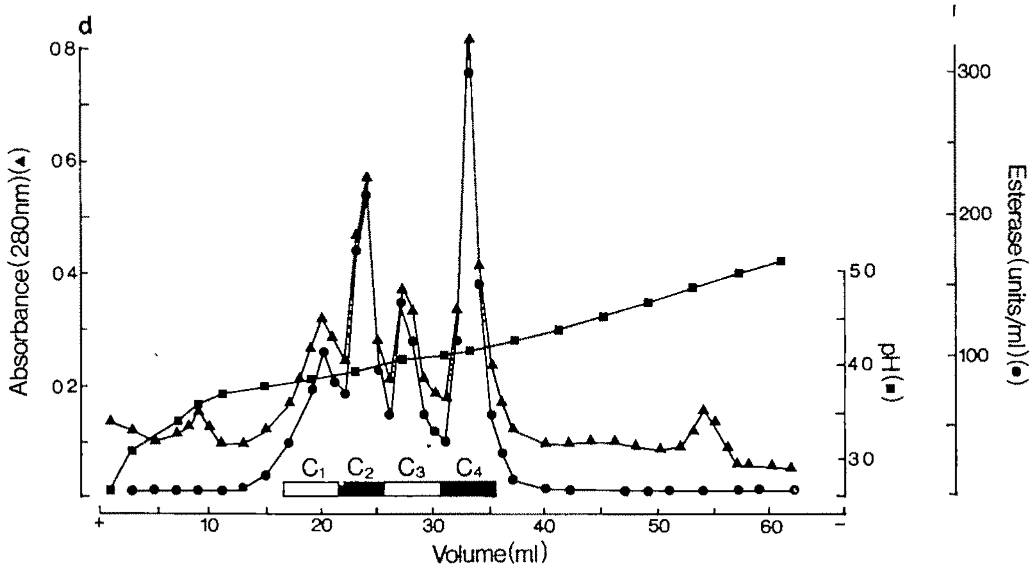


Figure 1 Purification of rat submandibular gland kallikreins, esterases C_1–C_4. (a) DEAE-Sephadex A-50 chromatography. Column: 1.5 x 74 cm. Sample: 476 mg of Fraction 1 (Table 1) in 70 ml. Eluents: 300 ml equilibration buffer (0 10 M NaCl–0.01 M Na-phosphate, pH 6.0), and a 2000 ml linear gradient to 0.50 M NaCl–0.01 M Na-phosphate, pH 6.0. Flow rate: 19 ml/hour. Fraction volume: 6.5 ml. Temperature: 4°C. (b) Hydroxyapatite chromatography. Column: 0.70 x 29 cm. Sample: 22 mg of Fraction 2C (Table 1) in 6.0 ml. Eluents: 50 ml equilibration buffer (3 M NaCl–0.005 M Na-phosphate, pH 6.0), and a 200 ml linear gradient to 3 M NaCl–0.05 M Na-phosphate, pH 6.0. Flow rate: 2.6 ml/hour. Fraction volume: 2 6 ml. Temperature· 20°C. Horizontal bars indicate the pools collected.





(c) Bio-Gel P-150 gel filtration. Column: 1.5 × 91 cm. Sample: 4.2 mg of Fraction 3C (Table 1) in 1.3 ml equilibration buffer (1 M NaCl–0.01 M Na-phosphate, pH 7.4). Flow rate: 13 ml/hour. Fraction volume: 3.3 ml. Temperature: 4°C (d) Electrofocusing. Column: 110 ml containing 0.8% Ampholine pH 3–5. Sample: 4.2 mg of Fraction 3C (Table 1) in 1.0 ml 0.01 M Na-phosphate, pH 7.4. Voltage: 200 V/3 h, 400 V/17 h, and 800 V/4 hour. Elution flow rate. 120 ml/hour. Fraction volume· 1.0 ml. Temperature. 4°C. Horizontal bars indicate the pools collected contained 0.8% (v/v) Ampholine pH3–5. After focusing four esterase peaks were obtained with pI values: 3.87, 3.96, 4.07 and 4.16 (Figure 1d). These were collected, dialysed against 3 M NaCl–0.01 M Na-phosphate (pH 6.0), concentrated by pressure dialysis, and equilibrated against 0.01 M Na-phosphate (pH 7.4) to give Fractions $5C_1$–$5C_4$ (Table 1). Their specific activities were 5–19% lower than that of Fraction 3C. Thus the kallikreins were slightly unstable in the electrofocusing conditions used.

Purification of esterases B

The esterases B_1 and B_2 (Figure 1a) were purified by exactly the same procedures as the kallikreins (Figure

Table 1 Purification of rat submandibular gland kallikreins, esterases C_1–C_4

Step	*Treatment*	*Protein (mg)*	*Specific activity (EU*/mg)*	*Purification*	*Recovery (%)*
1	Submandibular gland homogenate, centrifuged extracted and dialysed to give fraction 1	476	144		
	Esterase C activity†		97	(1)	(100)
2	DEAE-Sephadex A-50 chromatography of fraction 1 to give fraction 2C	56	633	6.5	74
3	Hydroxyapatite chromatography of 22 mg of fraction 2C to give fraction 3C‡	26	738	7.6	42
4	Bio-Gel P-150 gel filtration of 4.2 mg of fraction 3C to give fraction 4C	2.8	726		
5	Electrofocusing of 4.2 mg of fraction 3C to give fractions:				
	$5C_1$ pI 3.87	0.39	603		
	$5C_2$ pI 3.96	0.50	700		
	$5C_3$ pI 4.07	0.48	610		
	$5C_4$ pI 4.16	0.77	636		

* EU (esterase unit) = 1.0 µmol of Bz-Arg-OEt/min (pH 8.5, 37 °C).
† Calculated on the assumption that 68% of the total esterase activity is due to esterase C, as found by DEAE-Sephadex A-50 chromatography.
‡ Protein and recovery have been calculated assuming that all of fraction 2C was fractionated.

Table 2 Purification of rat submandibular gland esterases B

Step	*Treatment*		*Protein (mg)*	*Specific activity EU*/mg*	*Purification*	*Recovery (%)*
1	Fraction 1 (see step 1, Table 1)					
	Esterase B activity†			31	(1)	(100)
2	DEAE-Sephadex A-50 chromatography of fraction 1 to give fractions	$2B_1$	24	288	9	44
		$2B_2$	12	342	11	29
3	Hydroxyapatite chromatography of fraction $2B_1$ to give fraction	$3B_1$	6.2	448	14	19
	fraction $2B_2$ to give fraction	$3B_2$	2.2	830	27	12
4	Bio-Gel P-150 gel filtration of fraction $3B_1$ to give fraction	$4B_1$	2.2	950	31	14
	fraction $3B_2$ to give fraction	$4B_2$	0.7	427‡	14	2

* EU (esterase unit) see Table 1.
† Calculated on the assumption that 21% of the total esterase activity is due to esterase B.
‡ The specific activity before concentration was 823 EU/mg.

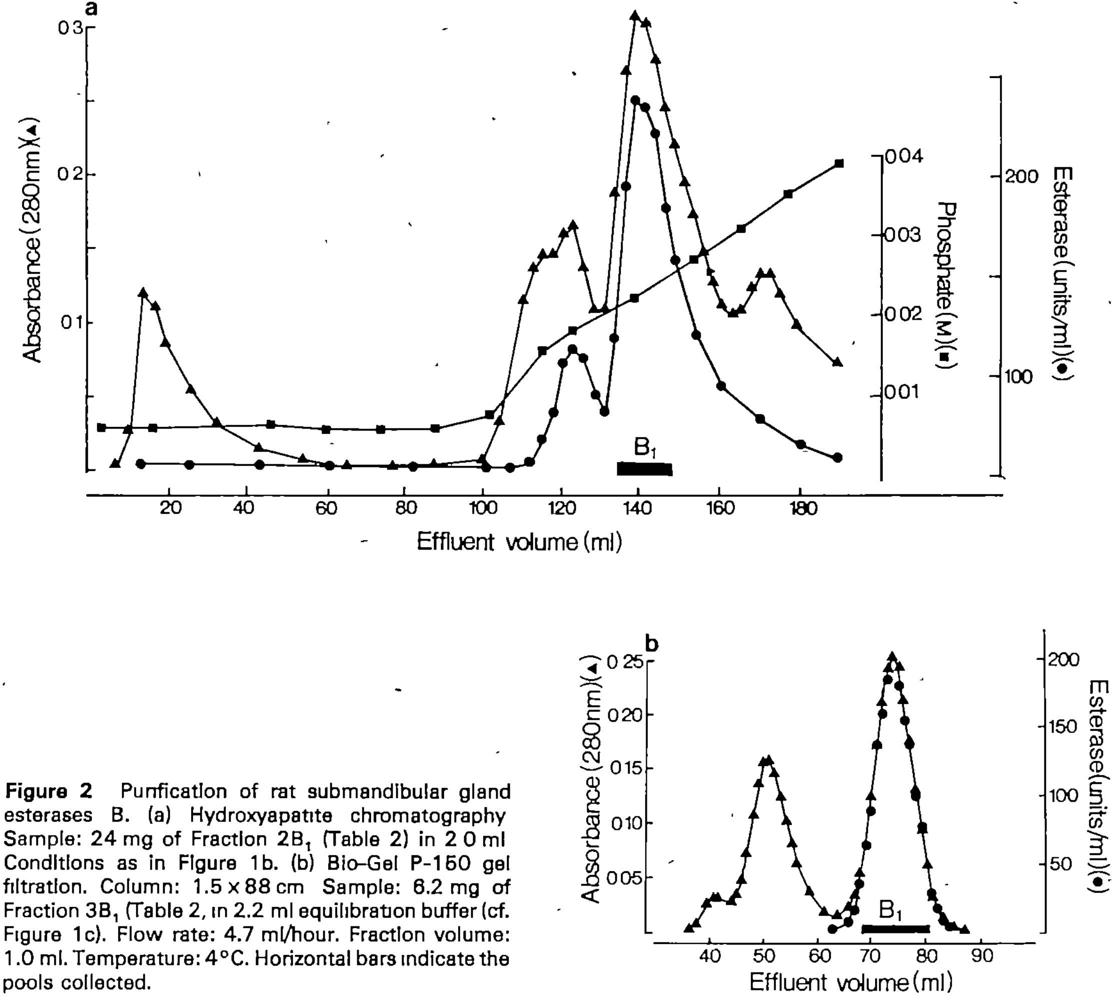


Figure 2 Purification of rat submandibular gland esterases B. (a) Hydroxyapatite chromatography Sample: 24 mg of Fraction $2B_1$ (Table 2) in 2 0 ml Conditions as in Figure 1b. (b) Bio-Gel P-150 gel filtration. Column: 1.5 x 88 cm Sample: 6.2 mg of Fraction $3B_1$ (Table 2, in 2.2 ml equilibration buffer (cf. Figure 1c). Flow rate: 4.7 ml/hour. Fraction volume: 1.0 ml. Temperature: 4°C. Horizontal bars indicate the pools collected.

2 and Table 2). Eighty per cent of esterase B_1 was eluted from the hydroxyapatite column at 0.022 M phosphate (Figure 2a), whereas 90% of B_2 was eluted at 0.028 M (data not shown). Esterases B_1 and B_2 were eluted from the Bio-Gel P-150 column at 1.81 and 1.84 times the void volume, respectively (Figure 2b). Only the protein peak corresponding to the esterase activity was found when B_2 was gel filtered (data not shown). The esterases B were unstable after gel filtration (Table 2) and electrofocusing in gradients of pH 3–10 and 4–6 caused more than 75% loss of enzyme activity. Fraction $4B_1$ focused as one major peak with a pI value of 5.30, and several small peaks at pH 4.84, 5.12, 5.55 and 5.70 (data not shown). Fraction $4B_2$ gave one major peak at pH 5.10 with a shoulder at 4.8 (data not shown).

Purification of esterases A

The esterase activity not adsorbed by DEAE-Sephadex A-50 was collected as two fractions, A_1 and A_2 (Figure 1a), concentrated by pressure dialysis, and equilibrated against 0.15 M NaCl–0.01 M Na-phosphate (pH 7.4). These fractions contained 39 and 9 mg protein and 95 and 214 EU/mg of esterase activity, respectively.

Polyacrylamide gel electrophoresis

Kallikreins (esterases C). Electrophoresis of different fractions (Table 1) in 16% polyacrylamide gels (Figure 3a) left kallikreins $5C_1$, $5C_2$ and $5C_3$ probably confined to single protein bands. However,

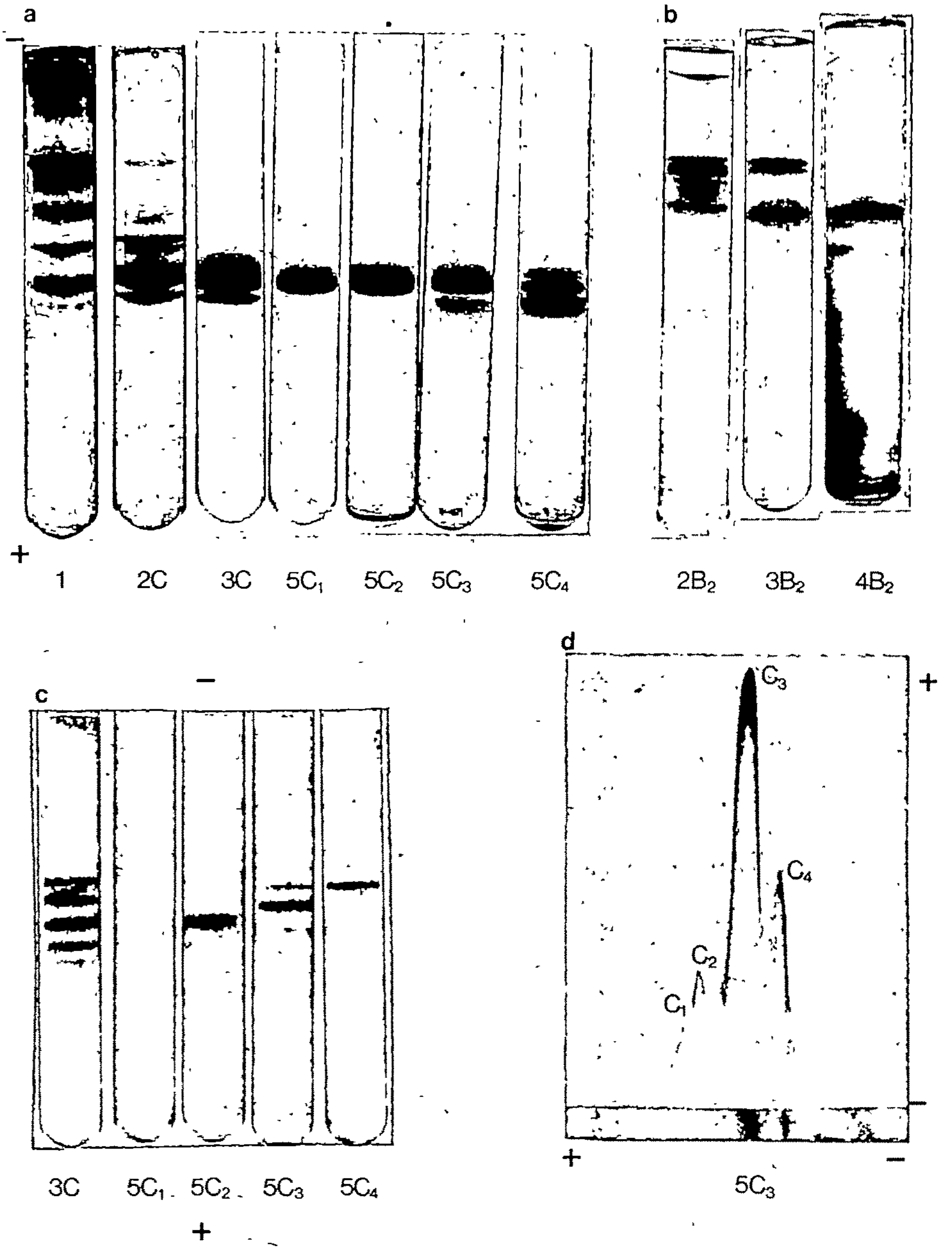


Figure 3 Electrophoresis (a and b) and electrofocusing (c and d) in polyacrylamide gels (a) Fractions from the purification of rat submandibular gland kallikreins (21–39 μg/gel) identified by their numbers in Table 1. (b) Fractions (see Table 2) from the purification of rat submandibular gland esterases B_2 (50 μg/gel). (c) Fractions from the purification of rat submandibular gland kallikreins (10–40 μg/gel) identified by their numbers in Table 1. Gel size 5 x 60 mm. (d) Fraction $5C_3$ (Table 1) 4 μg electrofocused in a polyacrylamide gel (2.7 x 70 mm) and transferred to crossed immunoelectrophoresis (see Methods section).

each fraction was contaminated by neighbouring fractions in the electrofocusing gradient (Figure 1c). The bands corresponding to C_1, C_2, C_3 and the slowest component of C_4 had similar mobilities, and constituted the wide protein band seen in all fractions prior to separation of C_1–C_4. Kallikrein C_4 appeared as two major bands and a slower minor one due to contaminating C_3. The fastest component of C_4 also appeared in Fractions 1, 2C, 3C and $5C_3$. Unstained gels of $5C_1$–$5C_4$ were sectioned, homogenized in 0.15 M NaCl–0.01 M Na-phosphate (pH 7.4). Assays of esterase activity showed a 60–70% recovery. It was located in the protein bands of $5C_1$–$5C_4$.

Esterases B. Electrophoresis in 16% polyacrylamide gels of esterases B_2 are shown in Figure 3b. Bz-Arg-OEt esterase activity was detected only in the fastest heavy protein band. Esterases B_1 were found in a protein band with the same electrophoretic mobility as esterases B_2 (data not shown).

Electrofocusing in polyacrylamide gel

The composition of kallikrein fractions 3C and $5C_1$–$5C_4$ are shown in Figure 3c. In addition to neighbouring kallikreins, fractions $5C_3$ and $5C_4$ contained 3 faint bands on either side of C_3 and C_4. These minor bands might represent kallikreins with different pI, since they reacted with the antiserum in crossed immunoelectrophoresis (Figure 3d).

Molecular weight determinations

Dodecyl sulphate polyacrylamide electrophoresis showed that each of the four kallikreins ($5C_1$–$5C_4$) gave a single band with identical mobility corresponding to a molecular weight of 34,000. A faster band of kallikrein C_4 (Figure 3a), visible with higher protein loads, may represent a kallikrein with a pI of 4.16, but with a smaller molecular weight.

Similar analysis of Fraction $4B_1$ showed a major protein band (calculated mol. wt. 37,500) followed by a minor impurity (data not shown).

Antigenic relationship between the kallikreins and other esterases

The antiserum to rat urinary kallikrein produced a reaction of identity between four urinary kallikreins, but did not react with a non-kallikrein esterase found in rat urine (Nustad & Pierce, 1974). This antiserum also reacted with the submandibular esterases A, B and, C. The immunoelectrophoretic patterns of rat submandibular extract (RSE; Fraction 1 in Table 1) and of esterases A, B and C, are shown in Figure 4a, b and c. The antigen in the major precipitin arc had a faster mobility than serum albumin, and corresponded to the four kallikreins C_1–C_4 (Figure 4a). The esterases B gave a continuous double arc merging with the cathodal part of the kallikrein line in a reaction of 'partial identity' (Figure 4a, b and c). Esterases A gave a continuous double arc and this was spurred over by the B-line (Figure 4c). When the antiserum was absorbed with esterases B only the C-line was retained (Figure 4a). Our interpretation of these results is summarized in Figure 4d.

In double diffusion tests with antiserum to rat urinary kallikrein (RUK) a reaction of identity was produced between that antigen (RUK) and submandibular gland extract (RSE) as well as between the four forms of esterase C (Figure 4e). The latter formed distinct spurs over the esterases A and B, thus confirming that A and B were antigenically deficient compared with kallikrein (Figure 4f). The two forms of esterase A, and the two forms of B, appeared antigenically similar, wherease the relationship between A and B could not be clearly established because of mutual contamination (Figure 4f). However, they clearly did not share all antigenic determinants.

Biological activity of submandibular gland esterases

The purified kallikreins $5C_1$–$5C_4$ released kinin equivalent to 42, 55, 36 and 42 µg of bradykinin min^{-1} mg^{-1} when incubated with rat kininogen. By contrast, the purified fractions of esterase B (Fractions $3B_1$ and $3B_2$, Table 2) and esterase A showed no detectable kinin releasing activity (far below 0.1 µg bradykinin mg^{-1} min^{-1}).

Each of the four kallikreins (1 µg of Fractions $5C_1$–$5C_4$) alone caused maximal contractions of the rat isolated uterus, comparable to effects of 0.2 µg of bradykinin. Esterases A and B had no effect in amounts 32 and 80 times those of esterases C. Intravenous injections of 0.1–0.3 EU of esterases C produced falls in the blood pressure of rats, 20–60 EU of esterases A caused small falls, whereas 20–40 EU of esterases B had no effect.

Caseinolytic activity of submandibular gland esterases

The caseinolytic activities expressed as caseinolytic units per Bz-Arg-OEt esterase unit (CU/EU) were: $5.2 \cdot 10^{-4}$ (esterases A); $2.6 \cdot 10^{-4}$ (esterases B); $1.6 \cdot 10^{-5}$ (Fraction 3C, Table 1); and $3.2 \cdot 10^{-1}$ (trypsin). The specific caseinolytic activities (CU/mg) were: 0.05 (esterases A); 0.08 (esterases B); 0.01 (3C); and 11.3 (trypsin).

Cellular localization of submandibular gland kallikreins

Very intense cytoplasmic fluorescence was localized in cells of granular tubules (Figure 5a). The cells were morphologically identified by examining neighbouring sections stained with haematoxylin and eosin, and subsequently by histochemistry on formalin-fixed tissue (Ørstavik, Brandtzaeg, Nustad & Halvorsen, 1975). The intensity of fluorescence decreased where the granular tubules merged with the striated ducts which showed only faint cytoplasmic staining except for a bright luminal rim (Figure 5a). Similar rims in striated ducts were the only fluorescent feature of

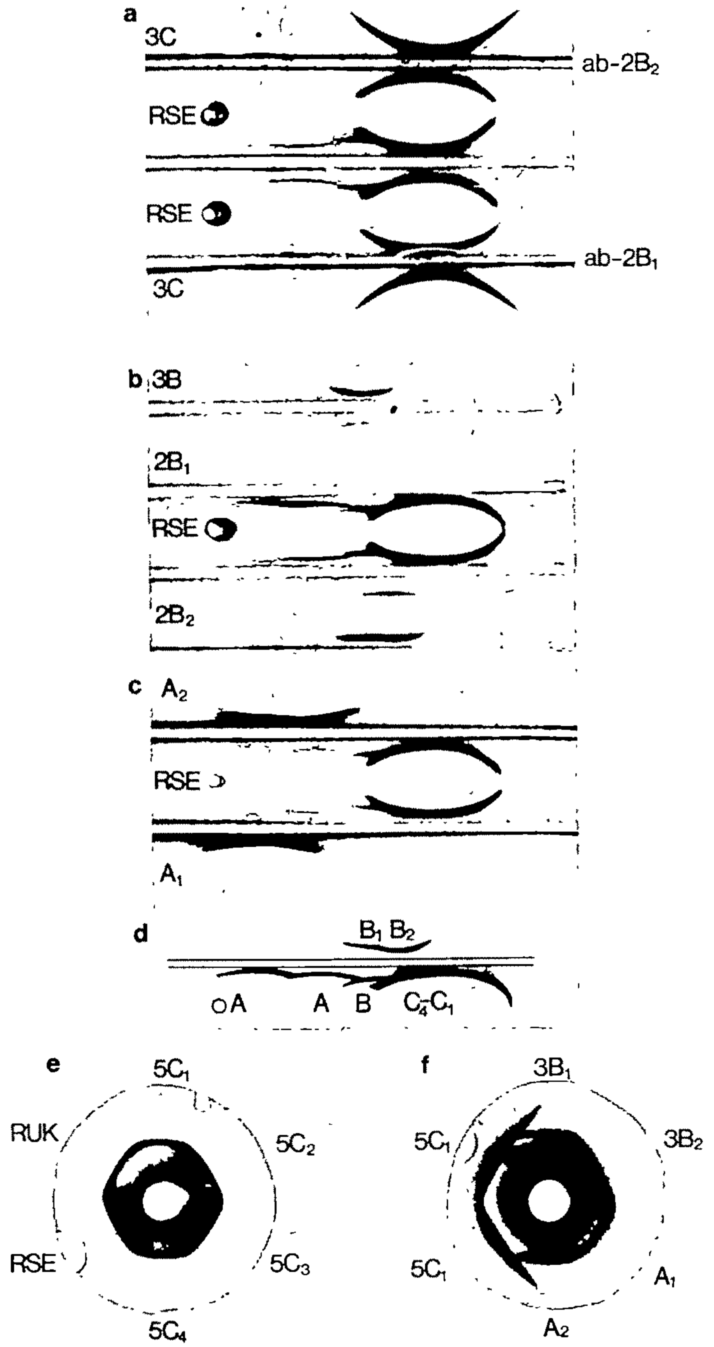


Figure 4 Immunoelectrophoresis and double immunodiffusion. The fractions are identified by their numbers (Tables 1 and 2). Rat submandibular extract (RSE) is a 30% (w/v) extract of the gland in 0.1 M NaCl–0.15 mM NaN_3–0.01M Na-phosphate, pH 7.4 (Fraction 1, Table 1, before addition of desoxycholate and dialysis at pH 6.0). Rat urinary kallikrein (RUK) B_3, Fraction 4C, Table 1, 1.0 mg/ml from Nustad & Pierce, 1974). The esterase A, B and C fractions (2–5 μl) were analysed at 0.5–1 mg/ml. Undiluted antiserum to rat urinary kallikrein (RUK) was used in all experiments except in Figure 4a where the middle trough contained antiserum diluted (1 : 2) with buffered saline, and the upper and lower troughs contained antiserum absorbed with 0.50 mg/ml of Fractions $2B_1$ and $2B_2$ (ab–$2B_1$ and ab–$2B_2$). (e) and (f) Double diffusion tests with antiserum to RUK, s[illegible]e text.

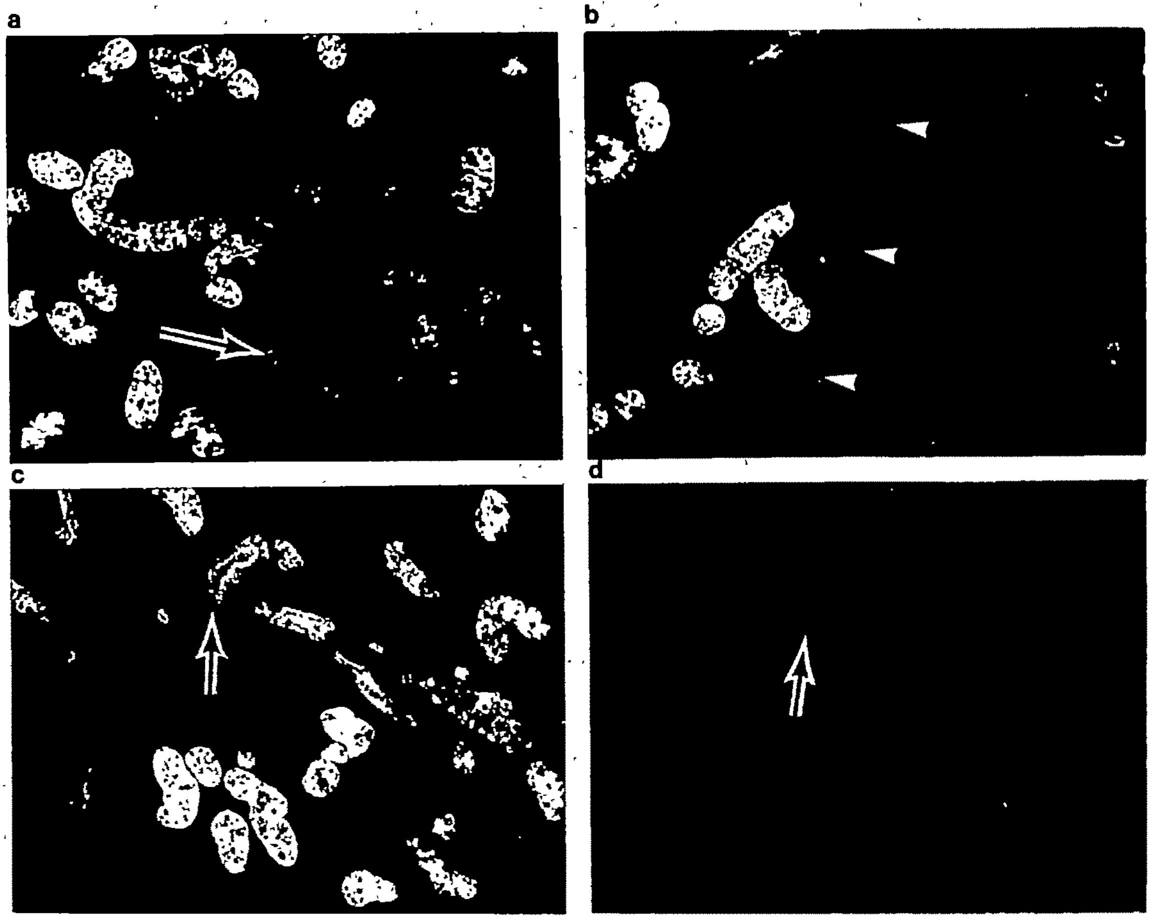


Figure 5 Immunohistochemical demonstration of kallikrein in alcohol-fixed sections of rat submandibular and sublingual glands. (a) Intense cytoplasmic immunofluorescence of submandibular granular tubules. Faint fluorescence of striated ducts, with a brigher luminal rim (arrow). (b) The border between submandibular (left) and sublingual (right) glands is indicated by arrow heads. Note four striated ducts with apical cytoplasmic fluorescence and absence of granular tubules in the sublingual gland. (c) and (d). Comparable fields in neighbouring submandibular sections incubated with antibody conjugate which had been absorbed with esterase B (c) or with esterase C (d). Esterase C abolished the staining reaction; the extremely faint fluorescence of ducts was revealed by overexposure. The arrow marks an identical site in the two pictures. Original magnification: 45×.

sublingual glands (Figure 5b). Faint cytoplasmic staining was seen in some larger ducts, but not in acini and stroma (Figure 5c).

Fluorescent staining was abolished by conjugate absorption with esterases C (Figure 5d), but not with esterases B (Figure 5c). This clearly demonstrated that the fluorescence specifically reflected the presence of kallikrein, and that the binding of the conjugate was specific (Brandtzaeg, 1973b). Specificity was substantiated by the extremely low staining end-point of the conjugate (4–8 µg/ml).

Discussion

The bulk of the high kallikrein content of the rat submandibular gland has been found in the granular tubules. A small amount was detected in the luminal border of striated duct cells. The latter is the main localization of kallikrein in the sublingual gland, which is devoid of granular tubules (Snell & Garrett, 1958). The granular tubules are differentiated from striated duct cells (Junqueira, 1967). Our results suggest that during their postnatal differentiation, granular tubules preserve the ability to produce kallikrein and acquire the ability to store it. The finding of kallikrein in tubules differs from the reported acinar localization in rat and mouse submandibular glands (Beraldo *et al.*, 1972; Bhoola *et al.*, 1973). Amongst enzymes found in granular tubules, is a trypsin-like enzyme which acts on ϵ-amino caproic acid naphthol ester (Lagunoff, Benditt & Watts, 1962). This substrate is probably not cleaved by kallikreins. In mouse granular tubules several antigenically similar Bz-Arg-OEt esterases have been demonstrated by indirect immunofluorescence (Ekfors & Hopsu-Havu, 1971; Ekfors, Malmiharju & Hopsu-Havu, 1972). They were characterized as plasminogen activators and permeability increasing enzymes, but not as kallikreins (Ekfors, Suominen & Hopsu-Havu, 1972). The nerve growth factor, another Bz-Arg-OEt esterase, is also found in the ducts in the mouse (Levi-Montalcini & Angeletti, 1961).

It is interesting that kallikrein, which is probably one of the regulators of local blood flow and membrane permeability (Hilton, 1970; Gautvik, 1970), has a different localization from the digestive enzyme amylase (Kraus & Mestecky, 1971). The large amount of kallikrein found in saliva strongly suggests an extracellular function. The ducts modify the primary saliva formed by the acinar cells (Schneyer, Young & Schneyer, 1972), and extracellular kinin formation may ensure adequate functional hyperaemia, and possibly facilitate transport of water, electrolytes and macromolecules across the tubular cells.

Werle, Vogel & Lendrodt (1960) reported that the rat submandibular gland was the richest source of kallikrein found, containing hypotensive activity of 3000 biological units/g wet wt., compared to 1–2 units/g wet wt. in man. This conclusion was questioned when it was reported that the main hypotensive enzyme in the rat submandibular gland and its saliva was a trypsin-like enzyme (salivain) which, unlike kallikrein, exhibited a strong caseinolytic activity (Riekkinen, Ekfors & Hopsu, 1966; Riekkinen & Ekfors, 1966; Ekfors, Malmiharju, Riekkinen & Hopsu-Havu, 1967a). Four kallikrein-like enzymes purified from the same gland had a low caseinolytic activity, but represented a minor part of the hypotensive activity of the gland (Ekfors *et al.*, 1967a, b). Our results clearly show that the main hypotensive enzymes in the gland can be identified as kallikreins by their kininogenase activity, and oxytocic activities, low caseinolytic activity, and their immunochemical identity with rat urinary kallikrein. We must therefore assume that salivain is a mixture of kallikreins and a caseinolytic enzyme. This was recently confirmed in experiments where a caseinolytic enzyme was found to be eluted together with the esterases C from DEAE-Sephadex A-50 columns, but could be separated by preparative disc gel electrophoresis (Vennerӧd, Laake & Nustad, unpublished observations). In the present work, this separation was obtained by hydroxyapatite chromatography.

The esterases B shared antigenic determinants with the kallikreins, and so did components present in the less pure fractions A, probably representing esterases A. The latter enzymes appeared antigenically even more deficient than the esterases B. These cross reactions may indicate that the esterases A, B and C all originate from a common trypsin-chymotrypsin like enzyme. We have not yet identified esterases A and B.

Requests for reprints to K.N. at The Norwegian, Radium Hospital.

References

ANDREWS, T. (1965). The gel-filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.*, **96**, 595–606.

BERALDO, W.T., SIQUEIRA, G., RODRIQUES, J.A.A. & MACHADO, C.R.S (1972). Changes in kallikrein activity of rat submandibular gland during post-natal development. In *Vasopeptides*, ed. Bach, N. & Sicuteri, F., pp. 239–250. New York: Plenum Publishing Corp.

BHOOLA, K.D., DOREY, G. & JONES, C.W. (1973). The influence of androgens on enzymes (chymotrypsin- and trypsin-like proteases, renin, kallikrein and amylase) and on cellular structure of the mouse submaxillary gland. *J. Physiol.*, **235**, 503–522.

BRANDTZAEG, P. (1970). *Human secretory immunoglobulins*. (Thesis). Oslo: Universitetsforlaget.

BRANDTZAEG, P. (1973a). Conjugates of immunoglobulins G with different fluorochromes. I. Characterization by anionic-exchange chromatography. *Scand. J. Immunol.*, **2**, 273–290.

BRANDTZAEG, P. (1973b). Conjugates of immunoglobulin G with different fluorochromes. II. Specific and non-specific binding properties. *Scand. J. Immunol.*, **2**, 333–348.

BRANDTZAEG, P. (1974). Mucosal and glandular distribution of immunoglobulin components. Immunohistochemistry with a cold ethanol-fixation technique. *Immunology*, **26**, 1101–1114.

DUNKER, A.K. & RUECKERT, R.R (1969). Observations on molecular weight determinations on polyacrylamide gel. *J. biol. Chem.*, **244**, 5074–5080.

EKFORS, T.O. & HOPSU-HAVU, V.K. (1971). Immunofluorescent localization of trypsin-like esteropeptidases in the mouse submandibular gland. *Histochem. J.*, **3**, 415–420.

EKFORS, T.O., MALMIHARJU, T. & HOPSU-HAVU, V.K. (1972). Isolation of six trypsin-like esteropeptidases from the mouse submandibular gland. *Enzymologia*, **43**, 151–165.

EKFORS, T.O., MALMIHARJU, T., RIEKKINEN, P.J. & HOPSU-HAVU, V.K. (1967a). The depressor activity of trypsin-like enzymes purified from rat submandibular gland. *Biochem. Pharmac.*, **16**, 1634–1636.

EKFORS, T.O., RIEKKINEN, P.J., MALMIHARJU, T. & HOPSU-HAVU, V.K. (1967b). Four isozymic forms of a peptidase resembling kallikrein purified from the rat submandibular gland. *Hoppe-Seyler's Z. physiol. Chem.*, **348**, 111–118.

EKFORS, T.O., SUOMINEN, J. & HOPSU-HAVU, V.K. (1972). Increased vascular permeability and activation of plasminogen by the trypsin-like esterases from the mouse submandibular gland. *Biochem. Pharmac.*, **21**, 1370–1373.

GAUTVIK, K. (1970). ***Studies on vasodilator mechanisms in the submandibular salivary gland in cats.*** (Thesis). Oslo: Universitetsforlaget.

GAUTVIK, K.M., KRIZ, M. & LUND-LARSEN, K. (1972). Plasma-kinin and adrenergic vasodilatation in the submandibular salivary gland of the cat. *Acta physiol. scand.*, **86**, 419–426.

HAGLUND, H. (1971). Isoelectric focusing in pH gradients—a technique for fractionation and characterization of ampholytes. *Methods Biochem. Anal.*, **19**, 1–104.

HILTON, S.M (1970). The physiological role of glandular kallikreins. In *Handbook of experimental pharmacology*, ed. Erdos, E.G. Vol. 25, pp. 389–399. Berlin: Springer-Verlag.

JUNQUEIRA, L.C.U. (1967). Control of cell secretion. In *Secretory mechanisms of salivary glands*, ed. Schneyer, L.H. & Schneyer, C.A., pp. 286–301. New York: Academic Press.

KRAUS, F.W & MESTECKY, J (1971). Immunohistochemical localization of amylase, lysozyme and immunoglobulins in the human parotid gland. *Archs oral Biol.*, **16**, 781–789.

LAAKE, K. & VENNERÖD, A.M. (1974). Factor XII-induced fibrinolysis: studies on the separation of prekallikrein, plasminogen proactivator, and factor XI in human plasma. *Thrombosis Res.*, **4**, 285–302.

LAGUNOFF, D., BENDITT, E.P. & WATTS, R.M. (1962). Histochemical study of esterases homospecific with trypsin. *J. Histochem. Cytochem.*, **10**, 672–673.

LAURELL, C.B (1966). Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. *Analyt. Biochem.*, **15**, 45–52.

LEVI-MONTALCINI, R. & ANGELETTI, P.U. (1961). Growth control of the sympathetic system by a specific protein factor. *Quart. Rev. Biol.*, **36**, 99–108.

LOWRY, O H., ROSEBROUGH, N.J., FARR, A L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

MALIK, N. & BERRIE, A. (1972). New stain fixative for proteins separated by gel isoelectric focusing based on Coomassie brilliant blue. *Analyt. Biochem.*, **49**, 173–176.

NUSTAD, K. & PIERCE, J.V. (1974). Purification of rat urinary kallikreins and their specific antibody. *Biochemistry*, **13**, 2312–2319.

NUSTAD, K, PIERCE, J.V. & VAAJE, K. (1975). Synthesis of kallikrein by rat kidney slices. *Br. J. Pharmac.*, **53**, 229–234.

ÓRSTAVIK, T.B., BRANDTZAEG, P., NUSTAD, K. & HALVORSEN, K.M. (1975). Cellular localization of kallikreins in rat submandibular and sublingual salivary glands. Immunofluorescent tracing related to histochemical characteristics. *Acta Histochem.*, **54**, 183–192.

RIEKKINEN, P.J. & EKFORS, T.O. (1966). Demonstration of a proteolytic enzyme, salivain, in rat saliva. *Acta chem. scand.*, **20**, 2013–2018.

RIEKKINEN, P.J., EKFORS, T.O. & HOPSU, V.K. (1966). Purification and characterization of an alkaline protease from rat-submandibular gland. *Biochim. Biophys. Acta*, **118**, 604–620.

SCHNEYER, L.H., YOUNG, J.A. & SCHNEYER, C.A. (1972). Salivary secretion of electrolytes. *Physiol. Rev.*, **52**, 720–777.

SNELL, R.S. & GARRETT, J.R. (1958). The effect of postganglionic sympathectomy on the structure of the submandibular and major sublingual salivary glands of the rat. *Z. Zellforsch*, **48**, 639–652.

WEBER, K, PRINGLE, J.R. & OSBORN, M. (1972). Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. *Meth. Enzymol.*, **26**, 3–27.

WEEKE, B. (1973). General remarks on principles, equipment, reagents and procedures. In *A manual of quantitative immunoelectrophoresis. Scand. J. Immunol.*, **2**, Suppl. 1, 15–35.

WERLE, E., VOGEL, R. & LENTRODT, J. (1960). Über den Kallikreingehalt der Glandula Submandibularis wahrend der Entwicklung einiger Säugerarten und nach Abbindung des Ductus Whartoni. *Arch. int. Pharmacodyn. Thér.*, **126**, 187–193.

WRIGLEY, C. (1968). Gel electrofocusing—a technique for analyzing multiple protein samples by isoelectric focusing. *Sci. Tools*, **15**, 17–23.

(Received 14 April 1975)

Br. J. Pharmac. (1976), **56**, 169–178

DUAL EFFECT OF α-ADRENOCEPTOR ANTAGONISTS IN RAT ISOLATED VAS DEFERENS

A. JURKIEWICZ & N.H. JURKIEWICZ
Department of Biochemistry & Pharmacology,
Escola Paulista de Medicina, Caixa Postal 20372, 01000 São Paulo, Brazil

1 In rat isolated vas deferens, the isotonic contractile responses to low doses of noradrenaline or adrenaline were antagonized, and those to high doses were potentiated, by yohimbine, piperoxan, phentolamine and tolazoline. Effects due to intermediate doses were not affected, or were potentiated within about 30 min, following an initial inhibition.

2 The α-adrenoceptor blockers thus caused a shift to the right and an increase of the maximum height of log dose-response curves of α-adrenoceptor stimulants. For a given dose of antagonist, the onset was slower for the potentiating than for the blocking effect.

3 The shift to the right induced by piperoxan and yohimbine on dose-response curves of noradrenaline and adrenaline was analysed with the Schild plot, and the slopes obtained, around 0.3, were lower than expected from receptor theory. When cocaine was used to block neuronal uptake, the slopes were close to 1.0.

4 The increase in maximum response to noradrenaline and adrenaline induced by α-adrenoceptor blockers was dependent on the time of incubation, on the dose of antagonist, and on the initial height of responses to the agonist. A less pronounced potentiation was also obtained when acetylcholine was used as agonist.

5 The findings are explained in terms of receptor theory as being due to a dual effect of α-adrenoceptor antagonists; competitive antagonism proper, which may be disclosed after blockade of neuronal uptake, and an interaction at a different locus, which results in potentiation of the effects of noradrenaline and adrenaline.

Introduction

It is known that yohimbine blocks adrenaline contractions in rat isolated vas deferens (Martins & Valle, 1939) and that antagonism of piperoxan towards (±)-noradrenaline follows receptor theory (Ariëns, 1967). It is apparently contradictory that these antagonists can potentiate the effect of sympathetic stimulation (Ohlin & Strömblad, 1963; Swedin, 1972) and that phentolamine can potentiate the effects of noradrenaline (Barnett, Greenhouse & Taber, 1968), in this preparation.

In the course of experiments performed on the rat vas deferens, it was observed that the reversible α-adrenoceptor antagonists piperoxan, yohimbine, phentolamine or tolazoline antagonized noradrenaline-induced contractions, but the effect was sometimes changed to potentiation within some minutes (Jurkiewicz, Jurkiewicz & Valle, 1971a). Since these findings could help to explain the conflicting reports described above, experiments were undertaken in order to analyse the phenomenon quantitatively.

Methods

Rat isolated vas deferens

Adult albino rats, 5 to 6 months old, were killed with an overdose of ether. The vasa deferentia were dissected from the surrounding tissues and the lumina carefully washed with a solution of the following composition (mM): NaCl 138, KCl 5.7, $CaCl_2$ 1.8, NaH_2PO_4 0.36, $NaHCO_3$ 15, dextrose 5.5, prepared in glass-distilled, deionized water. Each organ was mounted in a 10 ml chamber containing the solution at 30°C, aerated throughout the experiment. Contractions were recorded by means of isotonic levers with 6 × amplification and 1 g load.

Although single doses of active drugs were used in preliminary experiments, most of the work was based on the kymograph recording of cumulative dose-response curves as previously described (Jurkiewicz *et al.*, 1969). Before starting the actual experiments, the tissue was allowed to equilibrate for about 30 min, and 2 or 3 cumulative curves in response to noradrenaline,

adrenaline, or acetylcholine, were obtained at 20 to 60 min intervals, in order to achieve stable responses. Cumulative response curves to barium chloride were also obtained at the beginning and end of most of the assays. Except for barium chloride, only one agonist was used in each preparation. In some of the experiments, the contralateral vas deferens was used as an 'external control preparation' (Furchgott, 1970) in which cumulative curves in response to agonists were determined throughout the experiment, at similar intervals, in order to detect variations in sensitivity of the organ to the active drugs.

Four dose-response curves to an agonist were usually determined at 30 to 60 min intervals, following incubation with a dose of antagonist for periods of 1, 10, 20 and 30 min, in each experiment. In other series of assays the time of incubation with antagonist was constant (30 or 60 min) and 3 different doses of antagonist were used.

Measurement of antagonism and potentiation

In some experiments the degree of antagonism or potentiation was simply measured by the change in contraction height after each dose of antagonist. In general, cumulative curves were assessed by the height of the maximum contraction and by estimating the dose of active drug inducing a 50% effect (ED_{50}). Antagonism induced by a given dose of α-adrenoceptor blocker was estimated by a dose-ratio (ED_{50} after antagonists ÷ ED_{50} before antagonist) representing an apparent increase of ED_{50}. Potentiation was estimated by the ratio of the maximum response obtainable after, to that obtainable before, the addition of the potentiating drug.

Antagonism and potentiation in terms of receptor theory

In order to verify if antagonism followed receptor theory, plots were performed according to the relation (Schild, 1957), $\log(DR-1)=\log(B/K_B)$, in which DR is the dose-ratio as defined above, B is the molar concentration of antagonist, and K_B is the dissociation constant of the antagonist-receptor complex.

As an aid for the measurement of potentiation, the relative responsiveness ratio (ρ) was also estimated as the quotient between the height of maximum response to the agonist, and the height of the maximum contraction of the vas deferens, arbitrarily taken as that induced by barium chloride (Jurkiewicz, Jurkiewicz, Barros & Valle, 1969).

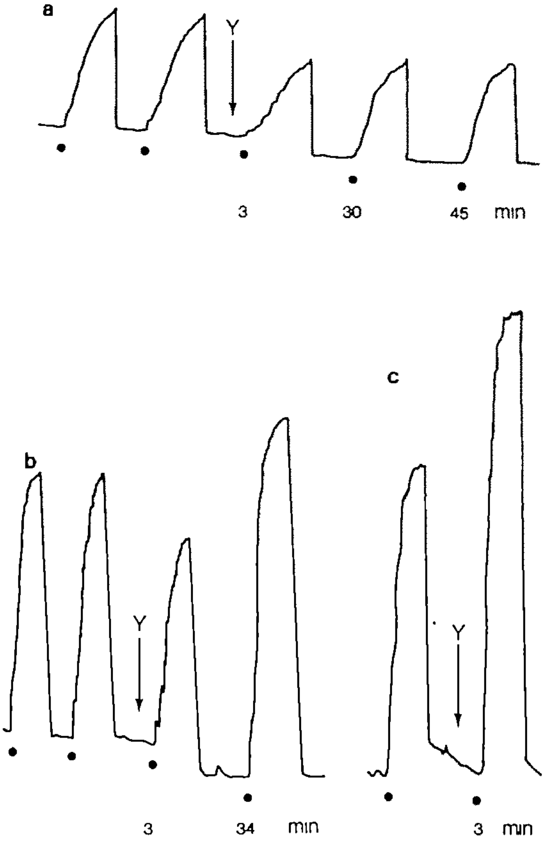


Figure 1 Effect of single doses of noradrenaline (at dots) on three different vasa deferentia (a,b,c), before and at different periods following addition of yohimbine 3×10^{-5} M (Y) to the nutrient solution Noradrenaline doses: (a) 3×10^{-6} M, (b) 3×10^{-6} M, (c) 1×10^{-4} M. After responses to noradrenaline (45 to 60 s) the Kymograph was stopped for agonist washout. Time in min after introduction of yohimbine solution is indicated. Besides its inhibitory or potentiating effect, yohimbine also induced a slight fall in resting tone.

Statistics

Significance of differences on the height of dose-response curves was analysed according to paired *t* test, slope of the lines on the Schild plot by a linear regression analysis and relations between relative responsiveness and potentiation by a correlation analysis (Snedecor & Cochran, 1967).

Drugs

(–)-Adrenaline ((–)-epinephrine bitartrate, Sigma, U.S.A., or Fluka, Germany), (–)-noradrenaline ((–)-arterenol hydrochloride, Sigma, U.S.A.), acetylcholine (acetylcholine iodide or chloride, Sigma, U.S.A.), priscoline (tolazoline, Sigma, U.S.A.), piperoxan (chlorhydrate of piperidinomethyl benzodioxane, Specia, France), yohimbine (yohimbine hydrochloride, Sigma, U.S.A.), regitine (phentolamine, Ciba, Switzerland), and barium chloride (May & Baker, U.SA.), were used. Stock solutions were maintained frozen and discarded after 15 days. Working solutions of adrenaline and noradrenaline were prepared shortly before the experiments. Except in preliminary experiments, disodium ethylenediaminetetraacetic acid (EDTA, 10 μg/ml) was added to stock and working solutions in order to prevent catalytic oxidation of noradrenaline and adrenaline by traces of heavy metals (Furchgott, 1955).

Results

At a concentration of 3×10^{-5} M, yohimbine induced either a decrease (Figure 1a), a potentiation (Figure 1c) or an inhibition followed by a potentiation (Figure 1b), of the contractile effects of single doses of noradrenaline. In some experiments this concentration of yohimbine did not influence noradrenaline effects at all. Similar results were obtained with piperoxan, 3×10^{-6}M as the antagonist or adrenaline as the agonist.

The analysis of changes induced in cumulative dose-response curves show (Figures 2a, b) that 3×10^{-5}M yohimbine in contact for 10, 20 or 30 min induced two clear effects: a reduction in the contractions elicited by the lowest doses of adrenaline (up to 70×10^{-7} M) and an increase in the response to the highest doses of agonist (Figure 2a). As a consequence, the lower part of the log dose-response curve to adrenaline was shifted to the right, and slope of the curve increased, and the maximum of the curve increased from 60% of that for barium chloride to nearly the same as the maximum response to barium (Figure 2b). A response to adrenaline which is insensitive to this concentration of yohimbine can be identified, represented by the point at which the curves for adrenaline in the absence and the presence of antagonist cross. Figure 3 shows that similar potentiations of the maximum response were obtained when the antagonists piperoxan, phentolamine, and tolazoline were used for 10, 20 or 30 minutes. When acetylcholine was used as the agonist, a potentiation of the maximum response could also be obtained, but to a lesser degree. On the other hand, no potentiation or antagonism could be recorded in 8 experiments in which propranolol in doses up to 10^{-7} M, was used instead of the α-antagonists.

Figure 4 shows the potentiation of effects of adrenaline after incubation with a lower dose of yohimbine (3×10^{-6} M), during 1, 10, 20 and 30 min, respectively. The development of potentiation of the maximum effect of the agonist is slower than in Figure 2b in which the effect of a dose 10 times larger of yohimbine was represented. The effect of yohimbine on a range of effects induced by six lower doses of adrenaline is also illustrated. After 1 min no potentiation occurs, but a slight antagonism can be already noticed for all the doses of adrenaline, except the highest (3×10^{-4} M). A slow increase in maximum effect of adrenaline occurs as the incubation with antagonist is prolonged, and as a consequence the initial antagonism shown for the effects of 3×10^{-6} and 10^{-5} M adrenaline is overcome by potentiation. This result corroborates the result shown in Figure 1b, in which an initial decrease of adrenaline effect was followed by potentiation.

Experiments were also performed with higher doses of yohimbine and piperoxan in order to see if the maximum reponse to catecholamines could be increased above that to barium. Although this effect could be obtained in individual experiments, in general the potentiations with 3×10^{-5} M yohimbine and 10^{-5}M piperoxan did not exceed the maximum response to barium. Higher doses induced smaller degrees of potentiation, and with concentrations of 3×10^{-4} or more of either yohimbine or piperoxan, the maximum response to adrenaline or noradrenaline was reduced.

The degree of potentiation for a given dose of antagonist and for a constant period of incubation varied inversely with the initial height of the dose-response curve to the agonist. The contraction obtained by saturation of α-receptors, represented in Figure 5 by the ρ ratio (Jurkiewicz *et al.*, 1969) attains an average value which is about 60% of the maximum contractile capability of the rat vas deferens to barium chloride. The individual ρ values vary about the mean with a minimum of about 25% and a maximum of 75%. Figure 5 shows that the highest potentiations were attained for the lowest dose-response curves to agonist and *vice versa.*

In order to see whether piperoxan and yohimbine actually behave as competitive antagonists, in spite of this potentiation, an analysis was made of changes, induced at equilibrium conditions, on the log dose-response curves to adrenaline and noradrenaline by a series of doses of antagonists. Such an experiment is illustrated in Figure 6 (left) in which the influence of potentiation was eliminated graphically by expressing each set of responses as percentage of its own maximum. The horizontal shift of the noradrenaline dose-response curve produced by a 10-fold increase in yohimbine concentration was less than one quarter of a log unit, though it should be about one log unit. This indicates that as well as potentiation another factor is involved, which modifies the horizontal shift of dose-response curves which would be produced by yohimbine if it was a simple competitive antagonist. Experiments were therefore performed in order to examine the shift of dose-response curves to noradrenaline, after blockade of neuronal uptake (Iversen, 1967) with 10^{-4} M cocaine. Unlike

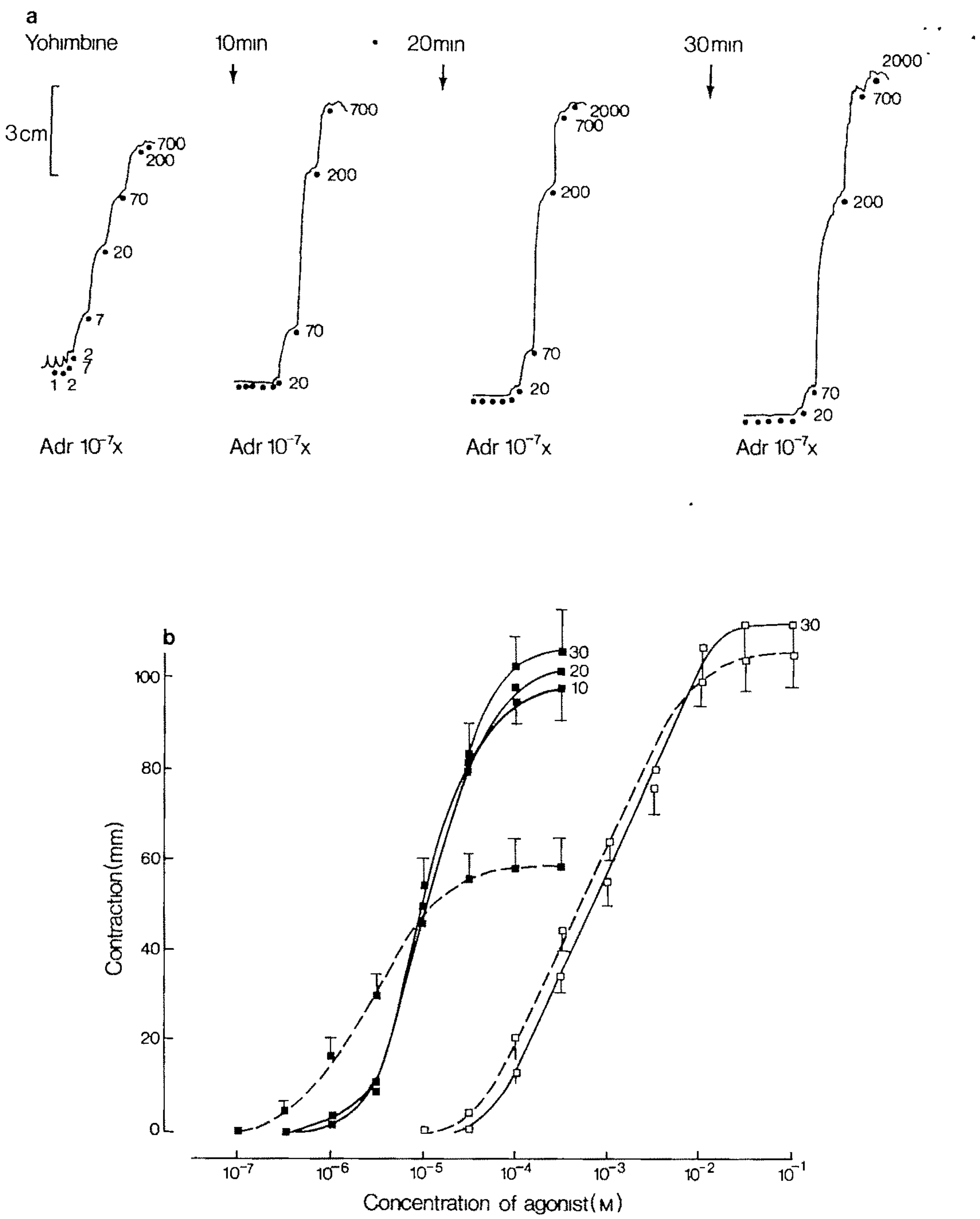


Figure 2 (a) Effect of cumulative molar concentrations of adrenaline (Adr) added at dots before and after 3 different periods of incubation with yohimbine 3×10^{-5} M (at arrows). Interval between curves was 30 to 45 minutes. (b) Mean log dose-response curves from experiments similar to (a). Responses to adrenaline (■); responses to barium (□) were tested at the beginning and end of each assay Control responses shown by broken lines; responses in presence of yohimbine, complete lines. Time of incubation with yohimbine shown (min) at end of each curve. Points represent means of 15 experiments. Vertical lines show s.e. mean.

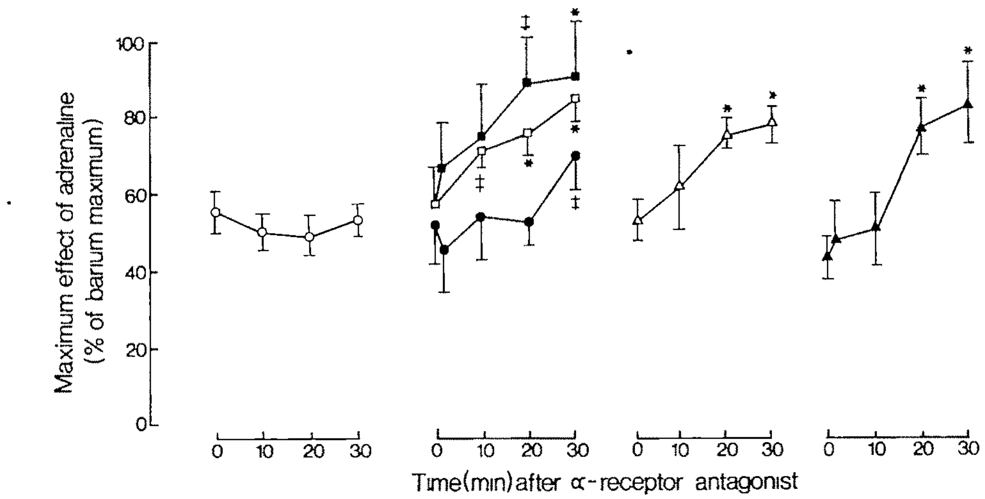


Figure 3 Variation in maximum cumulative effects of adrenaline, from experiments similar to that shown in Figure 2, after different periods of incubation without (O) and with competitive antagonists: piperoxan 3×10^{-8}, 3×10^{-8} and 3×10^{-8} M (●, ■, □), phentolamine 3×10^{-8} M (△) and tolazoline 3×10^{-8} M (▲). Maximum cumulative effect of barium chloride at the beginning of each experiment was taken as 100%. Each point is the mean of 4 to 11 experiments. Vertical lines show mean. ‡ $P<0.05$ and * $P<0.01$ compared to respective controls at 0 minutes.

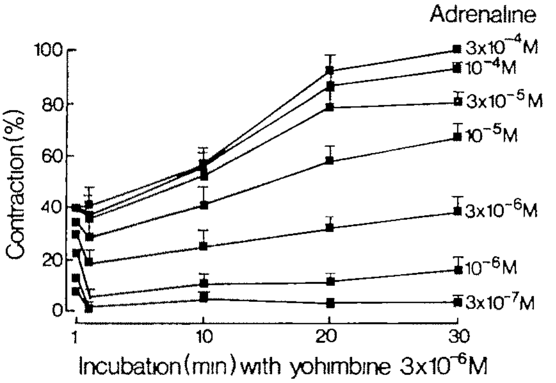


Figure 4 Variation in height of cumulative responses to adrenaline from experiments similar to that shown in Figure 2, after different periods of incubation with yohimbine 3×10^{-6} M. Maximum cumulative response to barium chloride at the beginning of each experiment was taken as 100% Results are means from 7 experiments. Vertical lines show s.e. mean.

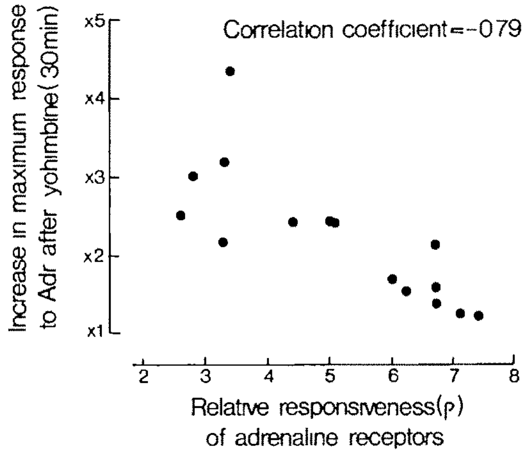


Figure 5 Negative correlation between potentiation induced by yohimbine 3×10^{-6} M, expressed as the ratio of the maximum cumulative responses to adrenaline before and after yohimbine, and the height of adrenaline control responses, expressed as the rho ratio. Each point represents a different experiment. The negative correlation is significant ($P<0.01$)

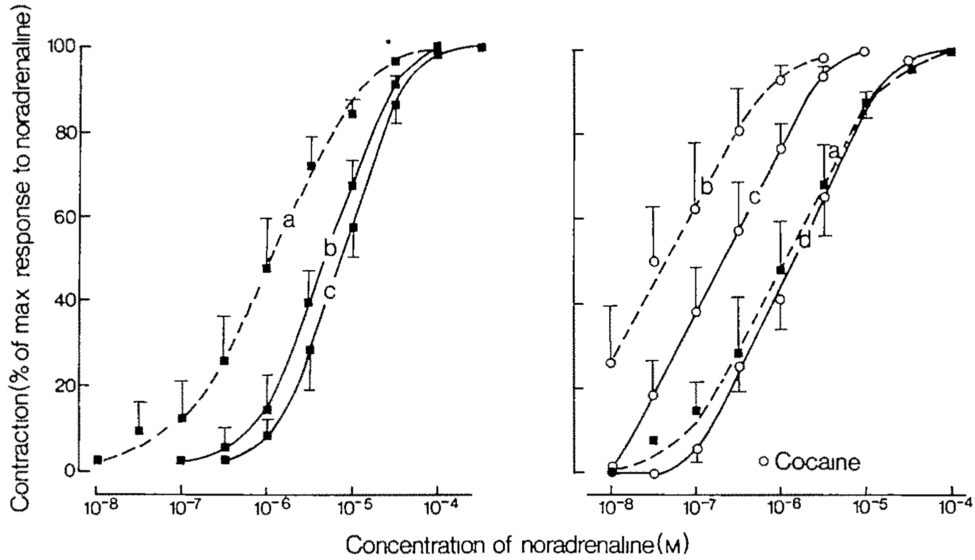


Figure 6 Mean cumulative dose-response curves for noradrenaline. Left panel—(a) control responses, (b) after yohimbine 3×10^{-8} M for 30 min, (c) after yohimbine 3×10^{-5} M for 30 minutes. Right panel—(a) control responses; cocaine 10^{-4} M was then added to the nutrient solution and maintained thereafter, (b) control responses, (c) after yohimbine 3×10^{-8} M for 30 min, (d) after yohimbine 3×10^{-8} M for 30 minutes. The effect of increasing the yohimbine concentration on the antagonism (b and c in the left panel) is greater in the presence of cocaine (c and d in the right panel). Means of 5 experiments. Vertical lines show s.e. mean.

lower and higher doses of cocaine, which can induce respectively an increase and a decrease of maximum effects of agonists (Ursillo & Jacobson, 1965; Kasuya & Goto, 1968), cocaine 10^{-4} M had no significant influence on the maximum response to noradrenaline, although it induced a shift of 1–1.5 log units to the left in the dose-response curve (Figure 6, right). After cocaine treatment the shift induced by a 10-fold increase in antagonist concentration was strikingly larger than before (Figure 6). The relation between the increment of antagonist concentration and the shift in dose-response curves was analysed by the Schild (1957) plot, according to which a slope of 1.0 is expected for the regression lines. Such a slope was obtained with antagonism of either noradrenaline or adrenaline by piperoxane, on cocaine-treated organs (Figure 7). This indicates that the antagonism exerted by the α-antagonists may be classified as competitive in rat vas deferens, though it is modified by potentiation and by the influence of removal mechanisms.

Discussion

It has been shown that so-called reversible α-adrenoceptor antagonists may either antagonize or potentiate the contractile effects of sympathomimetic agonists, depending on the doses and on the time of contact. Furthermore, it was also demonstrated that higher doses of α-blockers fail to cause a corresponding increase in the degree of antagonism (Figures 6 and 7), except when cocaine was simultaneously used. These results, when analysed on the basis of receptor theory, cannot be ascribed to a single mechanism of action of α-receptor antagonists, but indicate that several parts of the adrenaline receptor system are involved.

Adrenaline receptor system in rat vas deferens

Figure 8 pictures the receptor system as a chain of events, leading from drug-receptor interaction to the effect. It has been shown that this system is highly sensitive to the presence of calcium in the nutrient solution (Kasuya & Goto, 1968) and that more than one calcium kinetic compartment is probably involved (Jurkiewicz, Markus & Picarelli, 1975). Agonist concentration at the vicinity of receptors can be reduced by catalytic oxidation by traces of heavy metals present in the nutrient solution (Furchgott, 1955), or by removal mechanisms (Iversen, 1967; Furchgott, 1972). The possibility that sympathomimetic agonists interact with other sites besides the α-adrenoceptors proper cannot be excluded (Ganguly & Bhattacharya, 1969; Janis & Triggle, 1973).

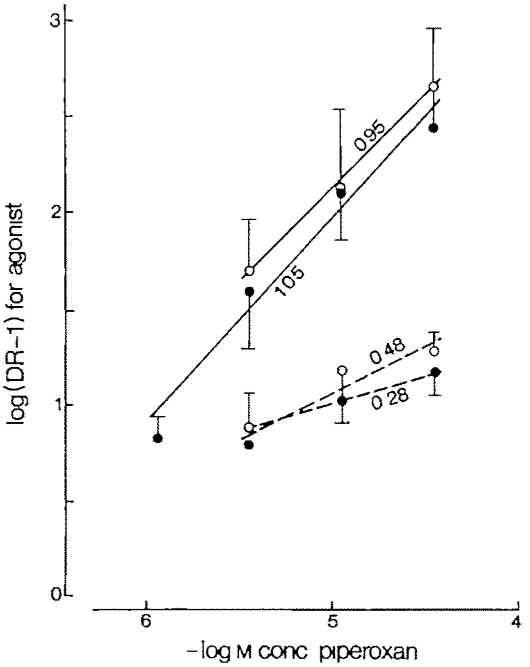


Figure 7 Schild (1957) plot for experiments similar to those shown in Figure 6. Piperoxan was used to antagonise adrenaline (O) and noradrenaline (●) with (full lines) or without (broken lines) 10^{-4} M cocaine. The slopes obtained for regression lines are indicated and were lower than the values expected according to receptor theory, except on cocaine-treated organs. Means of 3 to 5 experiments. Vertical lines show s.e. mean.

Competitive antagonism

According to occupation theory (see for example Ginsborg & Stephenson, 1974), log dose-response curves of agonists can be shifted to the right by reversible competitive antagonists, following Schild's (1957) equation. Shifts similar to those observed here, smaller than expected from theory (Figures 6 and 7), have also been described for other preparations, such as the cat nictitating membrane (Langer & Trendelenburg, 1969), and the guinea-pig trachea (Furchgott, Jurkiewicz & Jurkiewicz, 1973, 1974). A detailed review of the phenomenon was presented by Furchgott (1972). The theoretical approach assumes that a removal mechanism that can be saturated reduces agonist concentration in the biophase. The role of antagonists, by occupying α-receptors, is only to shift the range of doses of agonist up to concentrations high enough to approach saturation of the removal mechanisms. As a consequence, the relative concentration of agonist in the biophase increases and counteracts the shift due to competitive antagonism. For instance, a 10-fold increase of agonist concentration in an organ bath may represent a 20-fold increase in the biophase. When cocaine blocks a source of removal, such as uptake$_1$ (Iversen, 1967; Iversen & Langer, 1969), an increase of agonist concentration in the biophase occurs. This is represented by a leftward shift of the log dose-response curve (Figure 6), and the shifts due to α-receptor antagonism are in accordance with Schild's equation (Figure 7).

The results described here, except for the potentiation, conform to the expectations based on current models for competitive antagonism associated with a

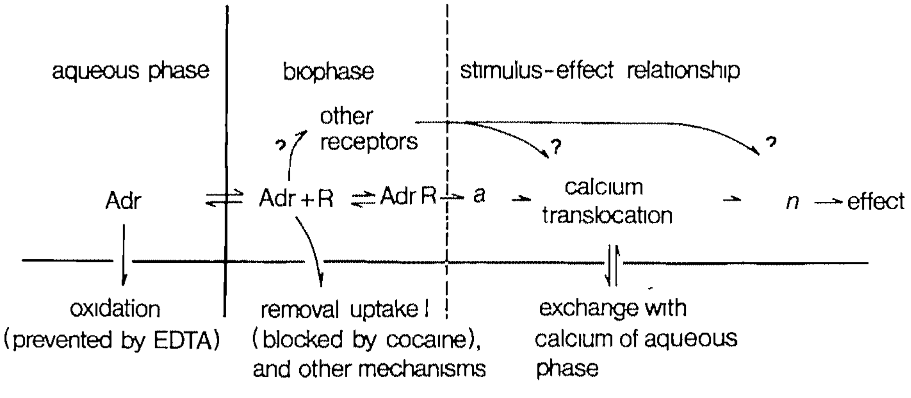

aqueous phase
biophase
stimulus-effect relationship
other receptors
?
?
?
Adr
Adr + R
AdrR
a
calcium translocation
n
effect
oxidation (prevented by EDTA)
removal uptake I (blocked by cocaine), and other mechanisms
exchange with calcium of aqueous phase

Figure 8 Schematic representation of adrenaline (Adr) receptor system in rat vas deferens, in which R represents α-receptors, AdrR the drug-receptor complex, and *a*.. *n* the steps leading from drug-receptor interaction to effect (Jurkiewicz, Jurkiewicz & Valle, 1971b). Step *a* represents the stimulus (Ariens, 1964). Once the stimulus is generated (*a* = efficacy × Adr R), the following steps (stimulus-effect relationship, after dotted line), are expected to occur independently of the drug efficacy. The biophase barrier (Furchgott, 1955), removal mechanisms (Furchgott, 1972), calcium translocation (Jurkiewicz, Markus & Picarelli, 1975) and other sites of interaction (Ganguly & Bhattacharya, 1969; Janus & Triggle, 1973) are indicated

neuronal uptake (Furchgott, 1972). Other possibilities that could be drawn from isolated experiments, as for instance from Figure 7, that the α-receptor blockers act as non-competitive antagonists because of the low slopes on the Schild plot, or that experiments similar to that shown in Figure 1b are due to desensitization to α-adrenoceptor blockers (Paton, 1967), are rendered unlikely by the overall analysis of our results. For instance, the shift induced on adrenaline curves (Figure 6) does not suggest a non-competitive antagonism, and effects similar to that of Figure 1b cannot be demonstrated by reducing or increasing the dose of agonist (Figures 1a, c).

Potentiation

Kalsner (1974) has recently suggested a classification of sensitization into two types: I, represented by a leftward shift of the log dose-response curve of agonist, and II, represented by an increase in maximum response. The potentiation induced by α-receptor blockers in rat vas deferens may thus be classified as type II sensitization.

In the light of receptor theory, potentiation may be caused by at least two groups of mechanisms (Figure 8): an increase of drug concentration in the vicinity of receptors through blockade of catalytic oxidation or of removal mechanisms, and alterations, either direct or indirect, in phenomena occurring after drug-receptor interaction. Increases of drug concentration in the biophase are bound to induce only type I sensitization (Furchgott, 1972), and may thus be ruled out in the analysis of the action of α-receptor blockers. The possibility remains that such an action is due to events occurring after drug-receptor interaction, though no conclusions can be drawn as to whether it occurs at the level of the stimulus-effect relationship, or on the capability of the drug-receptor complex to generate a stimulus, i.e. on efficacy (Ginsborg & Stephenson, 1974).

Figure 5 illustrates one of the limitations of type II sensitization: even with fixed conditions, as for instance after saturation of receptors with high doses of agonist, the degree of sensitization may vary according to the relative contractile capability of the receptor system. This result corroborates those of Westfall (1970) who found larger potentiations to occur for curves with lower slopes. The fluctuations on maximum effects of adrenaline and other *full* agonists, cannot be ascribed to variations in efficacy, since they could be due to variations in stimulus-effect relationship. Therefore, the ρ ratio is more appropriate to assess the maximum effect of this full agonist, but not of partial agonists, as previously discussed (Jurkiewicz *et al.*, 1969).

Since the initial scanty information concerning potentiation of sympathomimetic effects, including that induced by α-antagonists (Nickerson, 1949; Furchgott, 1955; Emmelin, 1961), several possibilities have been raised to explain the phenomenon (Kirpekar, Cervoni & Furchgott, 1962), mostly based on alterations in steps preceding drug-receptor interaction. In vas deferens, several drugs and procedures, as for example denervation, intervene in the stimulus-effect relationship (Ariëns, 1964; Kasuya, Goto, Hashimoto, Watanabe, Munakata & Watanabe, 1969; Westfall, 1970; Trendelenburg, 1972; Evans, Iwayama & Burnstock, 1973). It has been suggested that in some instances an alteration in the availability of calcium occurs. The view has also been advanced that irreversible α-adrenoceptor antagonists may interact directly with calcium binding sites (Janis & Triggle, 1973); it is known that an increase on the degree of sensitization may be attained by increasing calcium concentration in the aqueous phase from 1.7 to 8.0 mM (Kasuya & Goto, 1968).

The question now arises whether potentiation is unspecific (Fleming, 1971) or is actually related to α-antagonism. Although the different rates of onset of antagonism and potentiation indicate that these are two separate phenomena, the possibility that noradrenaline interacts with a second receptor population cannot be excluded. These are not β-adrenoceptors, since propranolol had no significant effect under our experimental conditions, but α-autoinhibitory sites (Barnett *et al.*, 1968). These sites could be slowly blocked by α-antagonists, indirectly causing a potentiation. The possibility of different types of α-receptors in a smooth muscle preparation has been previously suggested (Dale & Gaddum, 1930; Bentley & Smith, 1967; Furness, 1974) and would help to explain differences observed by application of endogenous and exogenous catecholamines (Swedin, 1972; Pennefather, 1973). The presence of regulatory α-receptors has been indicated in the case of catecholamine release due to nerve activity (Kirpekar, Wakade, Steinsland, Prat & Furchgott, 1972; Starke, 1972; Enero & Langer, 1973). If this is the case, it is difficult to explain why the effects of acetylcholine are also potentiated. The possibility that the potentiation is due to the release of catecholamines by acetylcholine is now under investigation.

Our thanks are due to Miss Haydee Reuter for technical assistance. This work was supported by the Brazilian Research Council (CNPq) and by FINEP.

References

ARIËNS, E.J. (ed) (1964). *Molecular Pharmacology, The mode of action of biologically active compounds*, Vol. 1, New York and London: Academic Press.

ARIËNS, E.J. (1967). The structure-activity relationships of *beta* adrenergic drugs and *beta* adrenergic blocking drugs. *Ann. N.Y. Acad Sci.*, **139**, 606–631.

BARNETT, A., GREENHOUSE, D.D. & TABER, R.I. (1968). A new type of drug enhancement: increased maximum response to cumulative noradrenaline in the isolated rat vas deferens. *Br. J. Pharmac.*, **33**, 171–176.

BENTLEY, G.A. & SMITH, G. (1967). Effects of alpha-adrenergic receptor blocking drugs on the response of vas deferens and arterial muscle to sympathetic drugs and stimulation. *Circulation Res.*, **20**, suppl. III, 101–110.

DALE, H.H. & GADDUM, J.H. (1930). Reactions of denervated voluntary muscle, and their bearing on the mode of action of parasympathetic and related nerves. *J. Physiol.*, **70**, 109–144.

EMMELIN, N (1961). Supersensitivity following "pharmacological denervation". *Pharmac. Rev.*, **13**, 17–37.

ENERO, M.A. & LANGER, S.Z. (1973). Influence of reserpine-induced depletion of noradrenaline on the negative feed-back mechanism for transmitter release during nerve stimulation. *Br. J. Pharmac.*, **49**, 214–225.

EVANS, B., IWAYAMA, T. & BURNSTOCK, G. (1973). Long-lasting supersensitivity of the rat vas deferens to norepinephrine after chronic guanethidine administration. *J. Pharmac. exp. Ther.*, **185**, 60–69.

FLEMING, W.W. (1971). Supersensitivity of the denervated rat diaphragm to potassium: a comparison with supersensitivity in other tissues. *J. Pharmac. exp. Ther.*, **176**, 160–166.

FURCHGOTT, R.F. (1955). The pharmacology of vascular smooth muscle. *Pharmac. Rev.*, **7**, 183–265.

FURCHGOTT, R.F. (1970). Pharmacological characteristics of adrenergic receptors. *Fedn. Proc.*, **29**, 1352–1361.

FURCHGOTT, R.F (1972). The classification of adrenoceptors (Adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Catecholamines, Handb. exp. Pharmac. N.S.*, Vol. 33, ed. Blaschko, H. & Muscholl, E., pp. 283–335. Berlin and Heidelberg: Springer-Verlag.

FURCHGOTT, R.F., JURKIEWICZ, A. & JURKIEWICZ, N.H. (1973). Analysis of propranolol antagonism to isoproterenol in guinea-pig trachea before and after blockade of active uptake. *Fedn. Proc.*, **32**, 723 Abs.

FURCHGOTT, R.F., JURKIEWICZ, A. & JURKIEWICZ, N.H. (1974). Antagonism of propranolol to isoproterenol in guinea-pig trachea: some cautionary findings. *Bioch. Pharmac.*, Suppl. pt 1, 220–224.

FURNESS, J.B (1974). Transmission to the longitudinal muscle of the guinea-pig vas deferens: the effect of pretreatment with guanethidine. *Br. J. Pharmac.*, **50**, 63–68.

GANGULY, D.K. & BHATTACHARYA, B.B. (1969). Adrenergic beta receptors in vas deferens. *Arch. int. Pharmacodyn. Thér.*, **185**, 406–412.

GINSBORG, B.L. & STEPHENSON, R.P. (1974). On the simultaneous action of two competitive antagonists. *Br. J. Pharmac.*, **51**, 287–300.

IVERSEN, L.L (1967). *The uptake and storage of noradrenaline in sympathetic nerves*. London: Cambridge University Press.

IVERSEN, L.L & LANGER, S.Z. (1969). Effects of phenoxybenzamine on the uptake and metabolism of noradrenaline in the rat heart and vas deferens. *Br. J. Pharmac.*, **37**, 627–637.

JANIS, R.A. & TRIGGLE, D.J. (1973). On the question of subclassification of α-adrenoceptors. *J. Pharm. Pharmac.*, **25**, 263–264.

JURKIEWICZ, A., JURKIEWICZ, N.H., BARROS, G.G. & VALLE, J.R. (1969). Relative responsiveness (ρ) of pharmacological receptor systems in the rat vas deferens. *Pharmacology*, **2**, 89–99.

JURKIEWICZ, N.H., JURKIEWICZ, A. & VALLE, J.R. (1971a). Aumento nos efeitos máximos de alguns agonistas pelo emprego de piperoxane e ioimbina no conduto deferente de rato. *Ciencia e Cultura (São Paulo)*, **23**, 384.

JURKIEWICZ, N.H., JURKIEWICZ, A. & VALLE, J.R. (1971b). Contractile reserve after maximum doses of full agonists on the rat vas deferens. *Pharmacology*, **5**, 129–144.

JURKIEWICZ, A., MARKUS, R.P. & PICARELLI, Z.P. (1975). Effect of full agonists following calcium deprivation in rat vas deferens. *Eur. J. Pharmac.*, **32**, 292–304.

KALSNER, S. (1974). A new approach to the measurement and classification of forms of supersensitivity of autonomic effector responses. *Br. J. Pharmac.*, **51**, 427–434.

KASUYA, Y. & GOTO, K. (1968). The mechanism of supersensitivity to norepinephrine induced by cocaine in rat isolated vas deferens. *Eur. J. Pharmac.*, **4**, 355–362.

KASUYA, Y., GOTO, K., HASHIMOTO, H., WATANABE, H., MUNAKATA, H. & WATANABE, M. (1969). Nonspecific denervation supersensitivity in the rat vas deferens *in vitro*. *Eur. J. Pharmac.*, **8**, 177–184.

KIRPEKAR, S.M., CERVONI, P. & FURCHGOTT, R.F. (1962). Catecholamine content of the cat nictitating membrane following procedures sensitizing it to norepinephrine. *J. Pharmac. exp. Ther.*, **135**, 180–190.

KIRPEKAR, S.M., WAKADE, A.R., STEINSLAND, O.S., PRAT, J.C. & FURCHGOTT, R.F. (1972). Inhibition of the evoked release of norepinephrine (NE) by sympathomimetic amines. *Fedn. Proc.*, **31**, 566 Abs.

LANGER, S.Z. & TRENDELENBURG, U. (1969). The effect of a saturable uptake mechanism on the slopes of dose-response curves for sympathomimetic amines and on the shifts of dose-response curves produced by a competitive antagonist. *J. Pharmac. exp. Ther.*, **167**, 117–142.

MARTINS, T. & VALLE, J.R. (1939). Endocrine control of the motility of the male accessory genital organs. *Endocrinology*, **25**, 80–90.

NICKERSON, M. (1949). The pharmacology of adrenergic blockade. *Pharmac. Rev.*, **1**, 27–101.

OHLIN, P. & STRÖMBLAD, B.C.R. (1963). Observations on the isolated vas deferens. *Br. J. Pharmac. Chemother.*, **20**, 299–306.

PATON, W.D.M. (1967). Adrenergic receptors viewed in light of general receptor theories. *Ann. N.Y. Acad. Sci.*, **139**, 632–644.

PENNEFATHER, J.N. (1973). A comparison of the responses of the isolated vas deferens of the rat to field stimulation and to noradrenaline added cumulatively and non-cumulatively. *Eur. J. Pharmac.*, **23**, 245–250.

SCHILD, H.O (1957). Drug antagonism and *pAx*. *Pharmac. Rev.*, **9**, 242–246.

SNEDECOR, G.W. & COCHRAN, G.C. (1967). Statistical methods. 6th ed. Iowa: Iowa State University Press.

STARKE, K. (1972). Influence of extracellular noradrenaline on the stimulation-evoked secretion of noradrenaline from sympathetic nerves: evidence for an α-receptor-mediated feed-back inhibition of noradrenaline release. *Naunyn-Schmiedeberg's Arch. Pharmac.*, **275**, 11–23.

SWEDIN, G (1972). Postnatal development of the mechanical response of the isolated rat vas deferens to nerve stimulation. *Acta physiol. scand.*, **84**, 217–223.

TRENDELENBURG, U. (1972). Factors influencing the concentration of catecholamines at the receptors. In *Catecholamines, Handb. exp. Pharmac. N.S.*, Vol. 33, ed. Blaschko, H. & Muscholl, E., pp. 726–761. Berlin and Heidelberg: Springer-Verlag.

URSILLO, R.C. & JACOBSON, J. (1965). Potentiation of norepinephrine in the isolated vas deferens of the rat by some CNS stimulants and antidepressants. *J. Pharmac. exp. Ther.*, **148**, 247–251.

WESTFALL, D.P. (1970). Nonspecific supersensitivity of the guinea-pig vas deferens produced by decentralization and reserpine treatment. *Br. J. Pharmac.*, **39**, 110–120.

(Received April 3, 1975.
Revised September, 1975)

Br. J. Pharmac. (1976), **56**, 179–186

THE EVALUATION OF THE NOVEL PRESSOR ACTIVITY OF γ-PIPERIDINOBUTYRAMIDE (WY 20051, DF480)

B.J. ALPS, P.W. DEVOY & J.F. WATERFALL
Department of Pharmacology, Wyeth Institute of Medical Research,
Taplow, Maidenhead, Berkshire

1 γ-Piperidinobutyramide (Wy 20051, DF480) injected intravenously evoked pressor responses in the anaesthetized ganglion blocked rat preparation over the dose range 2.4×10^{-6}–3.0×10^{-4} mol/kg.

2 High doses ($>3.8 \times 10^{-5}$ mol/kg) or even repeated submaximal doses (1.9×10^{-5} mol/kg) of Wy 20051 caused tachyphylaxis of this pressor response.

3 The noradrenaline pressor-response curve was shifted significantly to the right of the control curve following a dose of Wy 20051 (1.5×10^{-4} mol/kg cumulative).

4 The dose-response curve for the pressor action of Wy 20051 was potentiated in reserpine-treated anaesthetized rats. In contrast, tyramine-induced pressor responses were abolished.

5 Wy 20051 contracted the guinea-pig isolated aortic spiral preparation (3.8×10^{-5}–6.0×10^{-4} mol) and evoked constrictor responses in the perfused mesenteric vasculature preparation of the rat (5.9×10^{-7}–1.2×10^{-5} mol). At higher doses the responses were reduced.

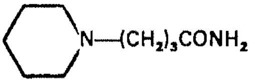
6 Wy 20051-induced constrictor responses of the perfused mesentery were unaffected by blockade of α-adrenoceptors or by tachyphylaxis of 5-hydroxytryptamine receptors.

7 The time for abolition of Wy 20051-induced constrictor responses of the mesentery in a calcium-free medium was not significantly different from that required for noradrenaline, but was significantly greater than that for KCl ($P < 0.001$).

8 Wy 20051 and noradrenaline, but not KCl, evoked constrictor responses in the depolarized rat mesenteric vasculature.

9 The results indicate that Wy 20051 evokes pressor responses which have some of the characteristics of those of noradrenaline. However, the responses are not elicited by an α-adrenoceptor mechanism.

Introduction



γ-Piperidinobutyramide (Wy 20051, DF480) a close derivative of γ-aminobutyric acid (GABA) was originally synthesized for comparative studies with the parent compound on the central nervous system. During examination of the pharmacology of Wy 20051 in the anaesthetized rat, it was observed that intravenous administration evoked transient pressor responses. The experiments described in this paper were designed to investigate the mode and site of action of this unusual pressor activity.

Methods

Intact animal studies

Female Charles River rats weighing 200–250 g were anaesthetized with pentobarbitone sodium (2.3×10^{-4} mol/kg i.p.). Each animal was allowed to breathe spontaneously through a tracheostomy tube. Drug-induced changes in blood pressure and heart rate were monitored by means of a Statham P23 pressure transducer from a catheter inserted into the left carotid artery and recorded on a Grass model 7 polygraph. Drugs were administered via a catheter into the jugular vein.

Ganglion blocked anaesthetized rats

A state of ganglion blockade was induced in each rat by administering pentolinium (2.0×10^{-5} mol/kg). The blockade was challenged with nicotine (6.2×10^{-7} mol/kg) 15 min after the administration of pentolinium and at intervals during the experiment. Further doses of pentolinium (8.4×10^{-6} mol/kg) were administered as required to maintain the blockade. A steady basal blood pressure was therefore established to facilitate measurement of pressor responses.

Dose-response curves to Wy 20051 (2.4×10^{-6}–3.0×10^{-4} mol/kg), noradrenaline (1.4×10^{-11}–1.5×10^{-8} mol/kg) or tyramine (1.8×10^{-8}–4.7×10^{-6} mol/kg) were obtained, followed by constant submaximal doses of each agonist (9.4×10^{-6} mol/kg, 2.4×10^{-9} mol/kg and 2.9×10^{-7} mol/kg respectively). In some experiments, dose-response curves to noradrenaline were obtained before and after a dose of Wy 20051 (1.5×10^{-4} mol/kg cumulative). In other experiments a constant submaximal dose of Wy 20051 (1.9×10^{-5} mol/kg) was administered every 7 min to anaesthetized rats and the effect on blood pressure recorded.

Reserpine-treated anaesthetized rats

Reserpine (8.2×10^{-6} mol/kg i.p.) was administered to rats 24 h before the start of the experiments. Only those animals in which tyramine (1.8×10^{-8}–4.7×10^{-6} mol/kg) did not evoke pressor responses were used. A dose-response curve to Wy 20051 (1.5×10^{-7}–3.8×10^{-5} mol/kg) was then obtained in the reserpine-treated rats.

Isolated tissue studies

All isolated tissues were suspended in (or perfused with) Krebs solution of the following composition (mmol/litre): NaCl 118.4 (13.0), KCl 4.7 (110.0), $NaHCO_3$ 25.0, KH_2PO_4 1.2, $CaCl_2$ 2.5 (0.25), $MgSO_4$ 1.2, glucose 11.0. The figures in parentheses refer to a depolarizing solution used in certain experiments. When a calcium-free Krebs solution was used $CaCl_2$ was omitted and 5.0×10^{-4} mol/litre ethyleneglycol bis-(aminoethyl)-tetracetic acid was added as a calcium chelating agent.

Where necessary, ascorbic acid (1.0×10^{-4} mol/litre) was added to the bathing fluid to prevent oxidation of noradrenaline. Organ bath volume was 35 ml. Perfusion rate for the perfused mesenteric vasculature preparation was 2 ml/minute.

Responses were recorded by variable inductance transducers connected to a phase discriminator and pen recorder or, in the case of the mesenteric vasculature preparation, pressure changes were recorded via a Bell and Howell L221-2-3 pressure transducer on a Devices M2 pen recorder.

The tissues were left to equilibrate for 1 h before dosing was started.

Guinea-pig aortic spiral

Contractions of the guinea-pig aortic spiral (Furchgott & Bhadrakom, 1953) were recorded (load 0.5 g) in response to doses of noradrenaline (1.1×10^{-7} mol) given at 5 min intervals until constant responses were obtained. The responses to Wy 20051 (3.8×10^{-5}–1.2×10^{-3} mol cumulative) or noradrenaline (1.2×10^{-9}–1.5×10^{-6} mol cumulative) were then observed.

Rat perfused mesenteric vasculature

The rat isolated perfused mesenteric vasculature preparation was set up as described by Collis & Alps (1973). Agonists were administered every 2 min or on restoration of basal perfusion pressure, whichever was the sooner. Constrictor responses were recorded as described.

Constrictor action of Wy 20051. Dose-response curves to noradrenaline (5.9×10^{-11}–4.7×10^{-8} mol) and constant responses to a submaximal dose (2.4×10^{-9} mol) were obtained. A response curve was then obtained to Wy 20051 (5.9×10^{-7}–1.2×10^{-4} mol).

Blockade of mesenteric constrictor responses. Approximately equal submaximal constrictor responses were obtained every 2 min with noradrenaline (1.2×10^{-9} mol), 5-hydroxytryptamine (5-HT, 4.5×10^{-9} mol), KCl (2.2×10^{-5} mol), $BaCl_2$ (9.6×10^{-6} mol) and Wy 20051 (4.7×10^{-6} mol). The tissue was then perfused with either (i) phenoxybenzamine (1×10^{-5} mol/litre); (ii) 5-HT (2.8×10^{-5} mol/litre); (iii) calcium-free Krebs solution or (iv) the depolarizing solution and the responses repeated. In some experiments with calcium-free Krebs solution the time taken for abolition of the mesenteric constrictor responses to noradrenaline, KCl and Wy 20051 was determined.

Effect of γ-aminobutyric acid. Doses of GABA (1.9×10^{-7}–1.9×10^{-4} mol) were given and the effect recorded.

α-Adrenoceptor blocking activity. Approximately equal submaximal constant responses were obtained to noradrenaline (4.7×10^{-9} mol) and KCl (1.1×10^{-5} mol). Bolus doses of Wy 20051 (4.7×10^{-5} mol) or phenoxybenzamine (3.3×10^{-10} mol) were given and 10 min later the responses to noradrenaline and KCl were repeated.

Drugs

Drugs used were (−)-ascorbic acid (BDH), ethyleneglycol bis-(aminoethyl)-tetracetic acid (Koch Light), 5-hydroxytryptamine creatinine sulphate (Koch Light), (−)-noradrenaline bitartrate (Koch Light), pentolinium bitartrate (May and Baker), phenoxybenzamine hydrochloride (Smith, Kline and French), reserpine (Ciba-Geigy) and tyramine hydrochloride (Koch Light). Drugs used in whole animal preparations were suspended in a 0.5% hydroxymethylcellulose-0.9% w/v NaCl solution

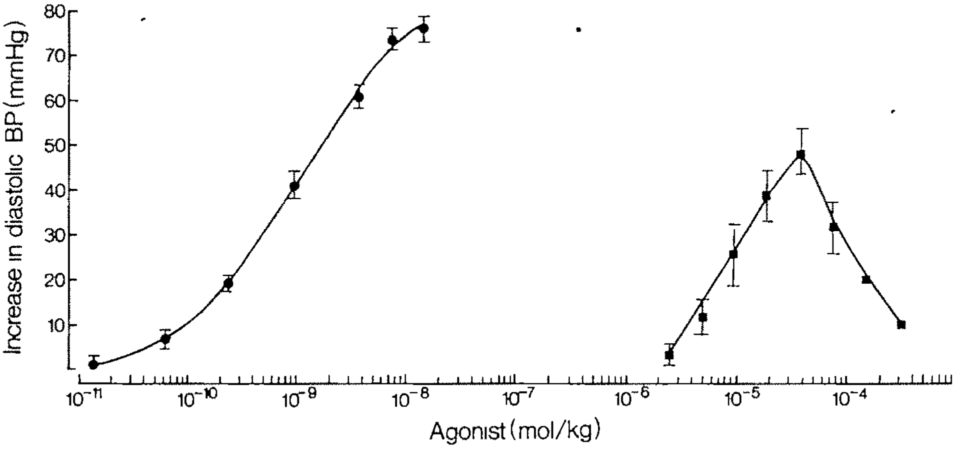


Figure 1 The effect of noradrenaline (●) and Wy 20051 (■) on the diastolic blood pressure of the anaesthetized ganglion blocked rat. Drugs were administered cumulatively i.v. via a cannula in the jugular vein. In contrast to noradrenaline high doses of Wy 20051 produced a diminished pressor response. Wy 20051 ($n=6$) was 1.28×10^4 times less potent than noradrenaline ($n=6$) when doses producing a 30 mmHg pressor response were compared.

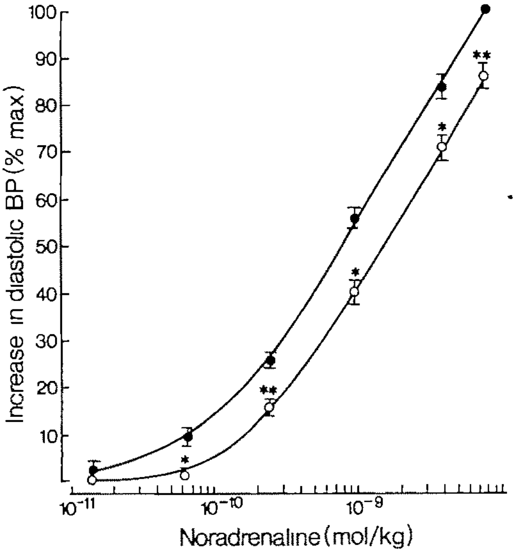


Figure 2 Response curves to noradrenaline before (●) and after (○) a cumulative dose of Wy 20051 (1.5×10^{-4} mol/kg $\simeq$ 3.4×10^{-5} mol) in anaesthetized ganglion blocked rats ($n=12$). Note that after Wy 20051 administration, the noradrenaline dose-response curve was significantly shifted to the right of the control curve. ★ $P<0.01$; ★★ $P<0.001$.

vehicle. Agonists used in isolated tissue preparations were dissolved in either Krebs solution (aorta) or distilled water (mesentery).

Results

Ganglion-blocked anaesthetized rats

Effect on diastolic blood pressure and heart rate. Wy 20051 transiently increased diastolic pressure over the cumulative dose range 2.4×10^{-6}–3.0×10^{-4} mol/kg. At higher doses ($>3.8\times10^{-5}$ mol/kg) these pressor responses were diminished (Figure 1). The maximum response to Wy 20051 (43.0 ± 5.8 mmHg; $n=6$) was significantly less than that to noradrenaline (75.0 ± 2.5 mmHg; $n=6$) ($P<0.001$). Doses of 9.4×10^{-6} mol/kg that had elicited pressor responses approximating to ED$_{30}$ before the response curve were ineffective when given after the response curve. At doses eliciting pressor responses, Wy 20051 had no effect on heart rate.

Effect of continual dosing. Continual administration every 7 min of a dose of Wy 20051 approximating to ED$_{50}$ (1.9×10^{-5} mol/kg) caused tachyphylaxis of the pressor responses. Responses were reduced to 42% of the starting level after the fourth dose and to 11% after the tenth dose ($n=6$).

Effect of Wy 20051 on noradrenaline dose-response curves. The noradrenaline dose-response curve was shifted significantly to the right of the control curve following a cumulative dose of Wy 20051 (1.5×10^{-4} mol/kg$\simeq3.4\times10^{-5}$ mol; Figure 2). The mean dose-ratio calculated at doses approximating to 30, 50 and 70% of the maximum response was 2.0.

Effect of tyramine on diastolic pressure. Tyramine produced a pressor effect within the dose range

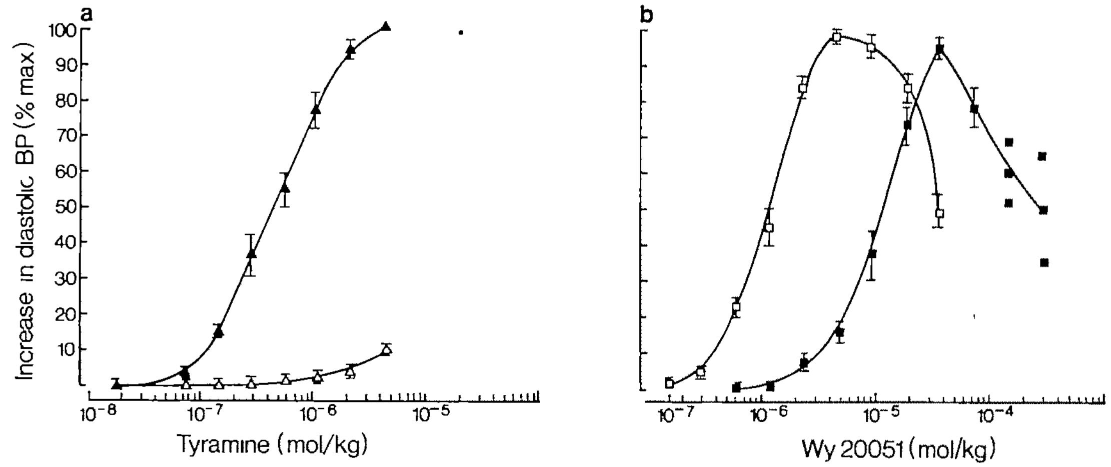


Figure 3 Effect of (a) tyramine and (b) Wy 20051 on the diastolic pressure of ganglion blocked (▲ and ■) and reserpine-treated (△ and □) rats. Rats were treated with reserpine or ganglion blocked as described in the text and tyramine ($n=6$) or Wy 20051 ($n=6$) given intravenously via a cannula in the jugular vein. Reserpine blocked tyramine-induced responses, but responses to Wy 20051 were potentiated.

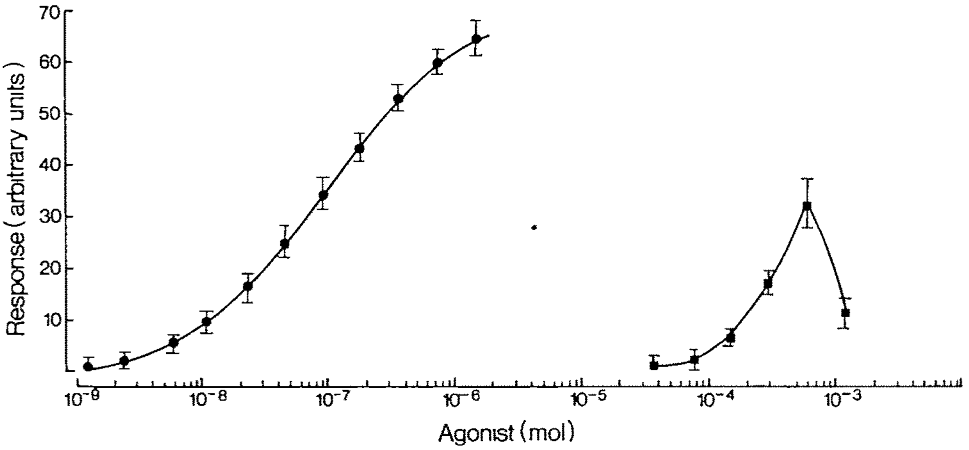


Figure 4 The constrictor activity of noradrenaline (●) and Wy 20051 (■) on the guinea-pig isolated aortic spiral preparation. The tissues were set up as described in the text. In contrast to noradrenaline high doses of Wy 20051 produced a diminished response. At the dose level producing a contraction of 30 arbitrary units, Wy 20051 ($n=5$) was 0.84×10^4 times less potent than noradrenaline ($n=5$).

1.8×10^{-8}–4.7×10^{-6} mol/kg (Figure 3a). Unlike Wy 20051, a constant submaximal dose of tyramine evoked equieffective pressor responses when administered after a dose-response curve and no diminution of responses was observed at high dose levels.

Reserpine-treated anaesthetized rats

The responses to tyramine previously obtained within the dose range 1.8×10^{-8}–4.7×10^{-6} mol/kg were not evoked in rats pretreated with reserpine (Figure 3a). In contrast, responses to Wy 20051 were potentiated in reserpine-treated animals (Figure 3b).

Guinea-pig aortic spiral

Wy 20051 (3.8×10^{-5}–6.0×10^{-4} mol cumulative) and noradrenaline (1.2×10^{-9}–1.5×10^{-6} mol cumulative) evoked contractions of the guinea-pig isolated aortic spiral. At higher doses of Wy 20051 (1.2×10^{-3} mol) the responses diminished (Figure 4).

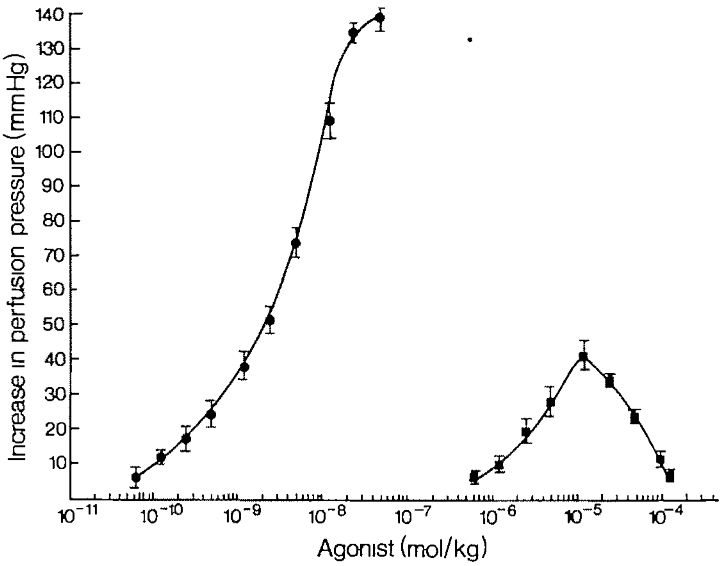


Figure 5 The constrictor activity of noradrenaline (●) and Wy 20051 (■) on the perfused mesenteric vasculature of the rat. The preparation was set up as described in the text. In contrast to noradrenaline, high doses of Wy 20051 produced a diminished constrictor response. At the dose level producing a 30 mmHg pressor response, Wy 20051 ($n=8$) was 0.82×10^4 times less potent than noradrenaline ($n=8$).

The maximum response to Wy 20051 (32.8 ± 5.0 arbitrary units; $n=5$) was significantly less than that produced by noradrenaline (65.0 ± 3.1 arbitrary units; $n=5$) ($P<0.001$).

The rat perfused mesenteric vasculature

Constrictor action of Wy 20051. Noradrenaline (5.9×10^{-11}–4.7×10^{-8} mol) and Wy 20051 (5.9×10^{-7}–1.2×10^{-4} mol) evoked constrictor responses of the perfused mesentery (Figure 5). The amplitude of the responses to Wy 20051 reached a maximum at 1.2×10^{-5} mol, but the maximum response (41.0 ± 4.3 mmHg; $n=6$) was significantly less than that produced by noradrenaline (137.0 ± 2.5 mmHg; $n=6$) ($P<0.001$). At higher doses the responses to Wy 20051 were reduced. The constrictor effect was absent at doses of 1.2×10^{-4} mol Wy 20051.

Phenoxybenzamine. In preliminary experiments phenoxybenzamine abolished noradrenaline-induced constrictor responses at a concentration of 1.0×10^{-7} mol/litre. Phenoxybenzamine (1.0×10^{-5} mol/litre) abolished responses to noradrenaline and 5-HT, but KCl, $BaCl_2$ and Wy 20051-induced constrictor responses were unchanged.

5-Hydroxytryptamine. Tachyphylaxis of mesenteric 5-HT receptors was produced by perfusing the tissue with 5-HT (2.8×10^{-5} mol/litre) in Krebs solution. An initial constriction occurred that gradually declined as the tachyphylaxis developed. When the perfusion pressure had returned to its basal value (approx. 1 h) the agonist sequence was repeated. The responses to noradrenaline, KCl, $BaCl_2$ and Wy 20051 were unchanged.

Calcium-free Krebs solution. Perfusion of the mesentery with calcium-free Krebs solution resulted in diminution and finally abolition of responses induced by noradrenaline, 5-HT, KCl and Wy 20051. Responses to $BaCl_2$ were unaffected. The mean ($n=5$)

Table 1 Abolition times for mesenteric pressor responses to submaximal doses of noradrenaline, KCl and Wy 20051 in the presence of calcium-free Krebs solution

Agonist	*Abolition time (min ± s.e. mean)*
KCl	47.4 ± 11.2
Wy 20051	97.8 ± 6.7
Noradrenaline	123.4 ± 9.2

Significance: KCl < Wy 20051 ($P<0.01$); KCl < noradrenaline ($P<0.001$); Wy 20051 *v.* noradrenaline NS.

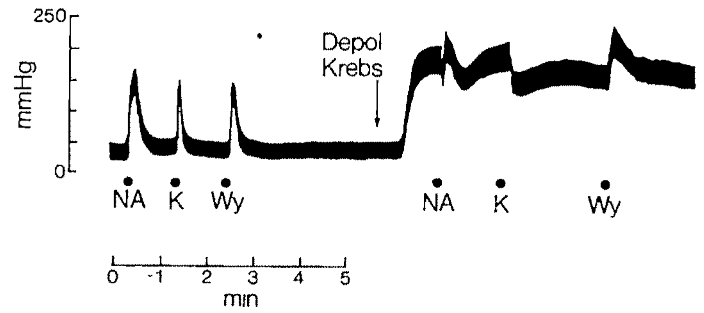


Figure 6 The effect of a depolarizing (high K^+) solution (see text) on mesenteric constrictor responses to submaximal doses of noradrenaline (NA, 2.4×10^{-9} mol), KCl (K, 1.1×10^{-8} mol) and Wy 20051 (Wy, 5.9×10^{-8} mol). Constant, approximately equieffective doses to the three agonists were obtained. The depolarizing solution induced a sustained contracture of the preparation. Under these conditions responses to KCl were abolished, but noradrenaline and Wy 20051 were still effective, although the responses were reduced

times taken for total abolition of responses to noradrenaline, KCl and Wy 20051 are summarized in Table 1.

Depolarizing solution. A sustained contraction of the mesenteric vasculature was induced by the depolarizing solution described in the methods section ($n=4$). Responses to doses of both noradrenaline and Wy 20051, which before depolarization had been equieffective, could be superimposed upon this contraction and were equally reduced in magnitude. In contrast no response was obtained to KCl (Figure 6).

Effect of γ-aminobutyric acid. The perfusion pressure of the isolated mesentery was unaltered by GABA (1.9×10^{-7}–1.9×10^{-4} mol).

α-Adrenoceptor antagonism. High doses of Wy 20051 (4.7×10^{-5} mol) did not reduce the response of the mesenteric vasculature to subsequent doses of noradrenaline (4.7×10^{-9} mol) or KCl (1.1×10^{-5} mol). However, a single dose of phenoxybenzamine (3.3×10^{-10} mol) abolished the responses to noradrenaline leaving those to KCl unchanged.

Discussion

Wy 20051 elicited pressor responses in the intact anaesthetized ganglion blocked rat, constrictor responses in the isolated perfused mesenteric vasculature preparation of the rat and evoked contractions of the guinea-pig isolated aortic strip. The possibility that Wy 20051 was indirectly stimulating α-receptors by causing release of noradrenaline at sympathetic nerve endings was discounted since Wy 20051-induced pressor responses were potentiated in reserpine-treated rats compared with normal control animals. In contrast tyramine-induced responses were abolished except at high doses where the effect could be ascribed to a direct action on the vascular smooth muscle (Krzanowski & Woodbury, 1966).

The reason for the potentiation of responses to Wy 20051 is not certain, but a non-specific supersensitivity to vasoactive agents other than catecholamines can result from reserpine treatment (Hudgins & Fleming, 1966). It is well established that catecholamine depletion is accompanied by a specific tissue supersensitivity to catecholamines (Burn & Rand, 1958; Shore, 1962) which may be caused, in the case of reserpine, by reduced overall catecholamine uptake, leaving more free catecholamine to react with tissue receptors. Trendelenburg (1966), whilst considering denervation supersensitivity in the nictitating membrane preparation, distinguished two components of supersensitivity, presynaptic and postsynaptic. Presynaptic supersensitivity is probably responsible for the specific effect on catecholamines whilst the non-specific supersensitivity for agents not taken up by adrenergic nerves such as peptides and, presumably, Wy 20051 may be due to vascular postsynaptic denervation supersensitivity. (Somlyo, Vinall & Somlyo, 1969.) Recent experiments show that reserpine increases the permeability of the muscle to calcium and acts, in addition, at some other calcium site to increase the availability of calcium for contraction (Carrier & Jurevics, 1973).

Direct stimulation of the α-adrenoceptor by Wy 20051 was considered to be unlikely since mesenteric constrictor responses to Wy 20051 could still be obtained in the presence of high concentrations of phenoxybenzamine (1.0×10^{-5} mol/litre); in contrast noradrenaline-induced responses were completely abolished by (1.0×10^{-7} mol/litre) phenoxybenzamine. Bevan, Osher & Su (1963) reported that high concentrations of phenoxybenzamine depressed responses of the rabbit aorta to K^+ probably by interference with transmembrane Ca^{2+} fluxes. This effect was not observed in the experiments on the mesentery although blockade of 5-HT-induced responses occurred (Nickerson, 1970).

Tachyphylaxis of the mesenteric 5-HT receptors to subsequent 5-HT-induced responses left Wy 20051-induced responses unchanged. It is therefore most unlikely that either α-adrenoceptor or 5-HT receptor stimulation is involved in the pressor action of this compound.

Perfusion of the mesenteric vasculature with calcium-free Krebs solution blocked noradrenaline, 5-HT, KCl and Wy 20051-induced responses, but did not inhibit $BaCl_2$-induced responses. $BaCl_2$-induced contractions seem to relate causally to inward movements of Ba^{2+}, which serve to function as a substrate for Ca^{2+} in the activation of contractile proteins (Toda, 1973). However, Ba^{2+} may also cause the release of Ca^{2+} (Bohr, 1964). The constrictor action of Wy 20051 unlike that of $BaCl_2$ seems to be calcium-dependent.

There is evidence that drugs can affect excitation contraction coupling by a common mechanism that is not entirely dependent on depolarization of the membrane. Thus noradrenaline responses on the rat tail artery persist long after the loss of the contractile response to high $[K^+]$ in a calcium-free medium (Hinke, 1965). In this series of experiments the time course for the abolition of Wy 20051-induced responses was significantly longer than that for KCl, but not significantly different from that of noradrenaline (Table 1). Furthermore, in the depolarized preparation, doses of noradrenaline and Wy 20051 that had evoked equal responses before depolarization continued to give equal, but reduced responses. However, responses could not be elicited for KCl (Figure 6). This phenomenon has been observed with maximal responses to different drugs in vascular smooth muscle (Somlyo & Somlyo, 1968a). These characteristics of pharmacomechanical coupling have been extensively reviewed by Somlyo & Somlyo (1968b, 1970) and have been ascribed to a longer and more persistent increase in the permeability of the membrane to calcium than is produced by depolarization, rather than an ability of the drugs to translocate Ca^{2+} into the cytoplasm from a compartment not accessible to depolarization. They proposed that membrane bound Ca^{2+} regulates the permeability of the membrane to ionized calcium itself. Depolarization removes some membrane bound calcium which causes increased permeability. Drugs acting by a pharmacomechanical mechanism release membrane bound calcium or eliminate its stabilizing effect with or without removing it from bound sites. The fact that noradrenaline produced consistently greater maximal responses than Wy 20051 both *in vivo* and *in vitro* suggests that noradrenaline was more able to overcome the stabilizing action of calcium and thereby produce a sustained increase in membrane permeability.

The tachyphylaxis of Wy 20051 pressor responses induced by high doses of the compound (3.8×10^{-5}–3.0×10^{-4} mol/kg) in intact animals is interesting since noradrenaline responses are also significantly reduced when preceded by doses of this order (3.4×10^{-5} mol; Figure 2). However, no demonstrable α-adrenoceptor antagonism could be detected in the isolated perfused mesenteric vasculature with even larger bolus doses of Wy 20051 known to produce tachyphylaxis in this preparation (4.7×10^{-5} mol; Figure 5). In contrast, a single bolus dose of phenoxybenzamine (3.3×10^{-10} mol) abolished the mesenteric pressor response induced by noradrenaline (4.7×10^{-9} mol). These experiments suggest that the reduction of Wy 20051 and noradrenaline responses *in vivo* occurring when these agonists are preceded by supramaximal doses of Wy 20051 do not directly involve the α-adrenoceptor. Possible explanations for this tachyphylaxis include exhaustion of bound calcium by Wy 20051, or permanent damage of the cell membrane with consequent disruption of calcium fluxes.

The chemical resemblance of Wy 20051 to GABA does not seem to be important in the cardiovascular activity of this compound since GABA had no effect on the rat mesentery and reports suggest that GABA has a depressor effect on blood pressure which is dependent on an optimal distance between the amino acid and carboxyl groups (Takahaski, Tiba, Iino & Takayasu, 1955). The depressor action of GABA in the rabbit has been ascribed to an action on the medulla (Takahaski, Tiba, Yamazaki & Noguchi, 1958) and in dogs to peripheral ganglion blockade (Stanton & Woodhouse, 1959; Stanton, 1963). However, pressor effects have been observed in the dog and may be due to carotid and aortic chemoreceptor stimulation.

In conclusion, Wy 20051 showed novel pharmacological activity in causing pressor responses in the intact animal and constrictor responses in the isolated perfused mesenteric vascular bed and guinea-pig isolated aorta. The contractions are calcium-dependent and are due, at least in part, to an action on pharmacomechanical coupling in the vascular smooth muscle.

The authors would like to thank Dr M.G. Collis for helpful discussion in preparation of the manuscript, Dr R. Crossley for the supply of Wy 20051 and Mrs M.A. Smith for excellent technical assistance.

References

BEVAN, J.A., OSHER, J.V. & SU, C. (1963). Response of vascular smooth muscle to potassium and its antagonism by phenoxybenzamine. *J. Pharmac. exp. Ther.*, **139**, 216–221.

BOHR, D.F. (1964). Contraction of vascular smooth muscle. *Can. med. Ass. J.*, **90**, 174–179.

BURN, J.H. & RAND, M.J. (1958). The action of sympathomimetic amines in animals treated with reserpine. *J. Physiol.*, **144**, 314–336.

CARRIER, O. & JUREVICS, H.A. (1973). The role of calcium in 'non-specific' supersensitivity of vascular smooth muscle. *J. Pharmac. exp. Ther.*, **184**, 81–94.

COLLIS, M.G. & ALPS, B.J. (1973). The evaluation of the α-adrenoceptor blocking action of indoramin, phentolamine and thymoxamine on the rat and guinea-pig isolated mesenteric vasculature and aortic spiral preparations. *J. Pharm. Pharmac.*, **25**, 621–628.

FURCHGOTT, R.F. & BHADRAKOM, S. (1953). Reactions of strips of rabbit aorta to epinephrine, isopropylarterenol, sodium nitrate and other drugs. *J. Pharmac. exp. Ther.*, **108**, 129–143.

HINKE, J.A.M. (1965). Calcium requirements for noradrenaline and high potassium ion contraction in arterial smooth muscle. In *Muscle*, ed. Paul, W.M., Daniel, E.E., Kay, C.M. & Monckton, G. pp. 269–285. London: Pergamon Press.

HUDGINS, P.M. & FLEMING, W.W. (1966). A relatively non-specific supersensitivity in aortic strips resulting from pretreatment with reserpine. *J. Pharmac. exp. Ther.*, **153**, 70–80.

KRZANOWSKI, J.J., Jr. & WOODBURY, R.A. (1966). Influence of tyramine on receptor interaction with dibenamine in aortic strips from reserpine-treated rabbits. *J. Pharmac. exp. Ther.*, **154**, 472–480.

NICKERSON, M. (1970). Drugs inhibiting adrenergic nerves and structures innervated by them. In *The Pharmacological Basis of Therapeutics*, ed. Goodman, L.S. & Gilman, A. pp. 550–557. London: Macmillan.

SHORE, P.A. (1962). Release of serotonin and catecholamines by drugs. *Pharmac. Rev.*, **14**, 531–550.

SOMLYO, A.P. & SOMLYO, A.V. (1968a). Vascular smooth muscle I. Normal structure, pathology, biochemistry and biophysics. *Pharmac. Rev.*, **20**, 197–272.

SOMLYO, A.P. & SOMLYO, A.V. (1970). Vascular smooth muscle II. Pharmacology of normal and hypertensive vessels. *Pharmac. Rev.*, **22**, 249–353.

SOMLYO, A.V. & SOMLYO, A.P. (1968b). Electromechanical and pharmacomechanical coupling in vascular smooth muscle. *J. Pharmac. exp. Ther.*, **159**, 129–145.

SOMLYO, A.V., VINALL, P. & SOMLYO, A.P. (1969). Excitation-contraction coupling and electrical events in two types of vascular smooth muscle. *Microvasc. Res.*, **1**, 354–373.

STANTON, H.C. (1963). Mode of action of gamma-amino-butyric acid on the cardiovascular system. *Archs int. Pharmacodyn. Thér.*, **143**, 195–204.

STANTON, H.C. & WOODHOUSE, F.H. (1959). The effect of gamma-amino-n-butyric acid and some related compounds on the cardiovascular system of anaesthetized dogs. *J. Pharmac. exp. Ther.*, **128**, 233–242.

TAKAHASKI, H., TIBA, M., IINO, M. & TAKAYASU, T. (1955). The effect of γ-amino-butyric acid on blood pressure. *Jap. J. Physiol.*, **5**, 334–341.

TAKAHASKI, H., TIBA, M., YAMAZAKI, T. & NOGUCHI, F. (1958). On the site of action of γ-amino-butyric acid on blood pressure. *Jap. J. Physiol.*, **8**, 378–390.

TODA, N. (1973). Influence of cadmium ions on the contractile response of isolated aortas to stimulatory agents. *Am. J. Physiol.*, **225**, 350–355.

TRENDELENBURG, U. (1966). I. Mechanisms of supersensitivity and subsensitivity to sympathomimetic amines. *Pharmac. Rev.*, **18**, 629–640.

(Received April 16, 1975
Revised September 16, 1975)

Br. J. Pharmac. (1976), **56**, 187–192

SODIUM AND CALCIUM IONS IN THE CONTROL OF TEMPERATURE SET-POINT IN THE PIGEON

P.N. SAXENA
Department of Pharmacology, J.N. Medical College, A.M.U., Aligarh, India

1 The effect of altering the ionic balance of the cerebrospinal fluid (CSF) on cloacal temperature of unanaesthetized pigeons kept at room temperature (20–25°C) was examined by injection or infusion of solutions of different ionic composition into a cannulated lateral cerebral ventricle.

2 An increase in the concentration of calcium ions caused a fall in temperature and behavioural sedation. The effects were the same whether the calcium was present as calcium chloride or as the calcium disodium salt of ethylenediamine tetra-acetic acid ($CaNa_2EDTA$).

3 When the concentration of sodium ions in the CSF perfusate was increased by addition of NaCl or that of calcium ions was decreased by addition of Na_2EDTA a rise in temperature was often produced but this was not consistent. NaCl sometimes had either no effect or lowered the temperature. Na_2EDTA while producing a rise when first injected failed to do so when repeated a few hours, 24 h and often 72 h later. Prolonged infusion of either agent caused intense behavioural excitement leading to death.

4 Potassium ions, like sodium ions, caused a rise in temperature but only when infused continuously. Behavioural excitement was only rarely observed.

5 Magnesium produced a fall in temperature. The concentration required was much higher than that of calcium but the hypothermia was more prolonged suggesting a slower elimination of the magnesium ions from the CSF. Magnesium ions caused tremors, nystagmus and ataxia as opposed to sedation caused by calcium.

6 All these were central effects as they were not obtained when the substances were injected intravenously.

7 Since changes in body temperature of the pigeon produced by injection of calcium or sodium ions into the CSF were similar to those seen in various species of mammal, it is concluded that the relative concentration of these ions within the brain plays an important role in establishing the temperature set-point in both birds and mammals.

Introduction

A change in the ratio of sodium to calcium ions in the artificial cerebrospinal fluid (CSF) perfused through the cerebral ventricles produces consistent changes in body temperature in the same direction in the cat (Feldberg, Myers & Veale, 1970; Myers & Veale, 1971), rabbit (Feldberg & Saxena, 1970a, b), monkey (Myers, Veale & Yaksh, 1971), rat (Myers & Brophy, 1971) and sheep (Myers, 1974). Feldberg *et al.* (1970) suggested that in homoeothermic animals the constancy of temperature depends on the balance of sodium and calcium ions in the anterior hypothalamus and that calcium ions may act as a kind of 'brake' and prevent the sodium ions from exerting their hyperthermic effect. According to Myers & Yaksh (1971), the animal retains its capacity to thermoregulate normally in either direction by mobilizing all the physiological systems for heat production or heat loss after a new set-point is reached following alteration of the sodium-calcium ratio. The set-point around which the body temperature is regulated depends on structures within the posterior hypothalamus in the cat (Myers & Veale, 1971) and the monkey (Myers & Yaksh, 1971) and can be reached by ions present in solutions perfused through the ventricles or microinfused into discrete areas in the posterior hypothalamus. The present study shows that the effects of sodium and calcium ions in determining set-point which have been observed in several species of mammal, also obtain in another class of homoeothermic animals, the birds.

Methods

Healthy pigeons of either sex and weighing 200–300 g were used. The right lateral ventricle was aseptically cannulated under intravenous pentobarbitone sodium anaesthesia according to the method described earlier

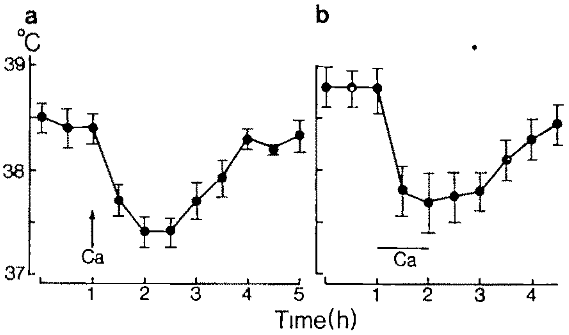


Figure 1 Mean cloacal temperature in unanaesthetized pigeons. At the arrow in (a) 0.02 ml of 1 04% $CaCl_2$ solution was injected into a lateral ventricle. The horizontal bar in (b) indicates a 60 min infusion of 0.2 ml of the same solution. The vertical bars indicate s.e. means of eight experiments in each of the two records.

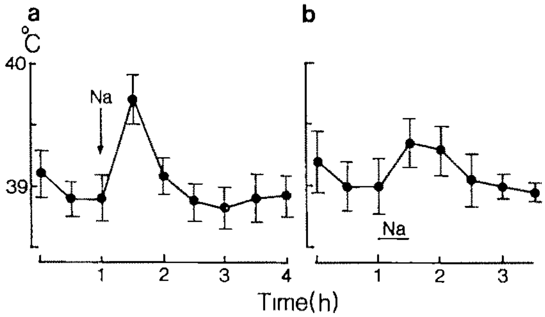


Figure 2 Mean cloacal temperature in unanaesthetized pigeons. At the arrow in (a) 0.02 ml of 0.9% NaCl solution was injected into a lateral cerebral ventricle. The horizontal bar in the right record indicates a 30 min infusion of 0.1 ml of the same solution. The vertical bars indicate s.e. means of 15 experiments in each group.

(Chawla, Johri, Saxena & Singhal, 1974). The pigeon was allowed two days to recover from the operation and thereafter used not more than twice a week for temperature study. The placement of the cannula, the zone of access of the injected solution and the ventricular contours were studied macroscopically at the end of a series of experiments in every pigeon, using bromophenol blue for vital staining.

Food and water were withheld during the period of experiment. The room temperature was maintained between 20–25°C. The pigeon's temperature was noted every 15 min from a multichannel telethermometer with the thermistor probe placed in the cloaca. The figures in this paper are based on these records.

The following solutions were employed for intraventricular or intravenous administration: pyrogen-free distilled water; artificial CSF of Merlis (1940) (composition: NaCl 8.1 g, 10% KCl 2.5 ml, 1 M $CaCl_2$ 1.27 ml, 1 M $MgCl_2$ 1.3 ml, 25% glucose 2.44 ml, urea 0.13 g, Na_2HPO_4 0.07 g, $NaHCO_3$ 1.76 g, water to 1000 ml); NaCl 0.9 and 1.5%; $CaCl_2$ 0.72, 1.04 and 1.45% (equivalent to 35, 50 and 70 times the concentration present in the artificial CSF); KCl 1.15% (equivalent to 46 times the concentration present in the artificial CSF); $MgCl_2$ 0.62 and 3.1% (equivalent to 50 and 250 times the concentration present in the artificial CSF); disodium salt of ethylenediamine tetra-acetic acid (Na_2EDTA) and calcium disodium salt of ethylenediamine tetra-acetic acid (Calcium Triplex-Merck; $CaNa_2EDTA$) 0.25 and 0.5%.

Glass articles, needles and tubes were made pyrogen-free before use by autoclaving and/or boiling. Pyrogen-free distilled water was used for preparation of the solutions.

Injections were made into the cannulated lateral ventricle by the technique described earlier (Chawla *et al.*, 1974). The volume of injection was 0.02 ml. For infusion of the solution into the lateral ventricle, a continuous slow injector was used in which was fitted a tuberculin syringe filled with the solution to be infused and connected to the protruding end of the intraventricular cannula by means of a 40 cm long polyethylene tube. The rate of infusion was 0.2 ml per hour. Intravenous injections were made into the pectoral vein and the volume of each injection was 0.1 ml.

Results

Injection or a 60 min infusion of distilled water or artificial CSF into a lateral cerebral ventricle did not influence the cloacal temperature of the pigeon. Thus, any dilatation of the ventricles caused by the injection procedure was of no consequence. Intravenous injections of 0.1 ml of these same solutions and of $CaCl_2$ (1.45%), NaCl (0.9%), KCl (1.15%), $MgCl_2$ (3.1%), Na_2EDTA (0.5%) and $CaNa_2EDTA$ (0.5%) were also without effect.

Calcium

Intraventricular injections of $CaCl_2$ solutions consistently produced a fall in cloacal temperature, the magnitude of which was in direct proportion to the concentration of the substance. Figure 1a shows the average effect in a group of pigeons of an injection of a 1.04% solution. In Figure 1b the effect of a 60 min infusion of the same solution is shown. The fall in

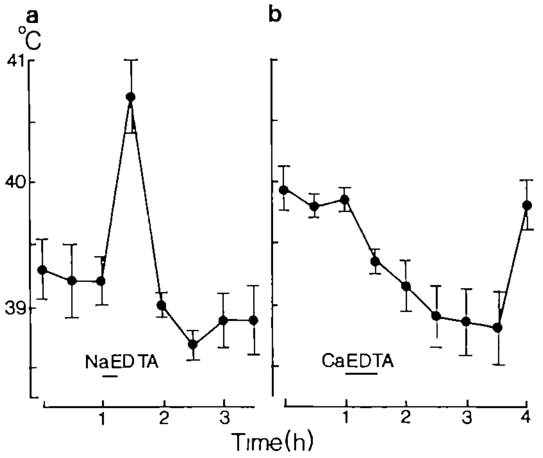


Figure 3 Mean cloacal temperature in un-anaesthetized pigeons. The horizontal bars indicate 0.2 ml per hour infusions of a 0.5% solution of Na_2EDTA (NaEDTA) (15 min) in (a) and of $CaNa_2EDTA$ (CaEDTA) (30 min) in (b). The vertical bars indicate s.e. means of eight experiments in each group. These were the first infusions of Na_2EDTA the pigeons had received.

temperature was about 1°C in each case and the time courses of the two responses were similar.

Infusion of a 1.45% $CaCl_2$ solution for the same length of time was followed by a profound fall in temperature and death several hours later in the three pigeons tested. However, pigeons receiving injections of the same solution survived.

All the pigeons were behaviourally sedated following administration of $CaCl_2$.

Sodium

Intraventricular injections or infusions of NaCl solutions produced only a mild effect on temperature but in the opposite direction to that of calcium. The record in Figure 2a shows the average rise in a group of pigeons following injection of 0.9% solution. The temperature rose by about 1.5°C following the injection and returned to normal within an hour. The pigeon became restless. It was not possible to sustain the rise even when the solution was infused for up to 30 minutes. On the other hand, such infusions produced a smaller rise. This is shown in Figure 2b. The large standard errors of the means (vertical bars) indicate that the rise was not a constant feature of intraventricular injections or infusions of NaCl, the temperature sometimes falling slightly below the original level. Infusion of a more concentrated solution (1.5%) over 30 min caused excessive motor excitement and resulted in death in 2 out of 3 pigeons.

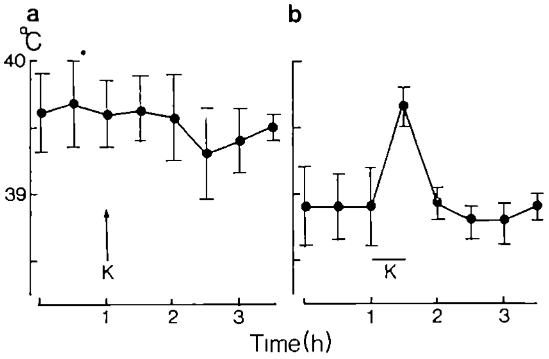


Figure 4 Mean cloacal temperature in un-anaesthetized pigeons. At the arrow in (a) 0.02 ml of 1.15% KCl solution was injected into a lateral ventricle. The horizontal bar in the right record indicates a 30 min infusion of 0.1 ml of the same solution. The vertical bars indicate the s.e. means of 10 experiments in each group.

EDTA

The effects of Na_2EDTA resembled those of NaCl presumably because it altered the sodium-calcium ratio in favour of sodium by chelating calcium ions. The first intraventricular injection of a 0.25 or 0.5% solution produced a rise in cloacal temperature which subsided within an hour. A second injection into the same pigeon on the same day or up to three days later often failed to produce the response. The rise in temperature could also not be sustained by infusing the solution. Figure 3a shows the average effect of a 0.5% solution infused for 15 min into a group of pigeons. In each case, this was the first infusion of Na_2EDTA that the pigeons had received. The sharp rise in temperature was followed by a slight short-lasting fall. When the infusion was prolonged to 25 min and beyond, intense motor excitement was produced and the pigeon died.

Intraventricular injections or infusions of $CaNa_2EDTA$ produced essentially similar effects on temperature to those obtained with $CaCl_2$ solution. Injections caused a dose-dependent fall in cloacal temperature. An infusion produced a larger and more sustained fall. Figure 3b illustrates the average fall in temperature in a group of pigeons following a 30 min infusion of a 0.5% solution of $CaNa_2EDTA$. The pigeons became sedated.

Potassium

Figure 4 illustrates the average effect on temperature of intraventricular injections (Figure 4a) and infusions (Figure 4b) of a 1.15% solution of KCl in two groups of pigeons. The injections were ineffective but the

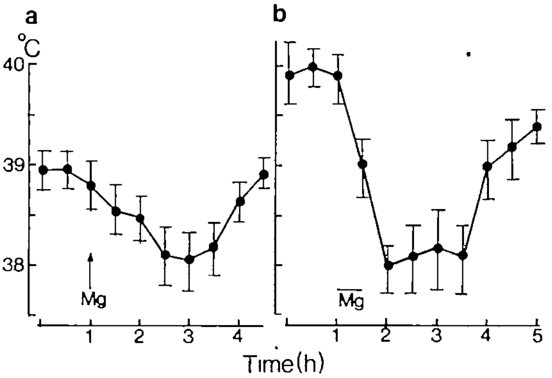


Figure 5 Mean cloacal temperature in unanaesthetized pigeons. At the arrow in (a) 0.02 ml of 3.1% $MgCl_2$ solution was injected into a lateral ventricle. The horizontal bar in (b) indicates a 30 min infusion of 0.1 ml of the same solution. The vertical bars indicate the s.e. means of 10 experiments in each group.

30 min infusions produced a rise which resembled that produced by injections of NaCl solutions (see Figure 2a). However, behavioural excitement was observed only in one out of ten experiments.

Magnesium

The effect on temperature of $MgCl_2$ resembled that of $CaCl_2$. Single intraventricular injections of $MgCl_2$ were relatively less effective than a continuous infusion (Figure 5). The record in Figure 5a shows the average effect in a group of pigeons of injections of a 3.1% solution of $MgCl_2$. The fall in temperature is somewhat smaller than that produced by injections of a 1.04% solution of $CaCl_2$ (Figure 1a). Upon infusion of these same solutions, however, it was observed that a 30 min infusion of $MgCl_2$ produced a more profound fall (2°C) than a 60 min infusion of $CaCl_2$ (1°C) (Figures 5b and 1b).

The behavioural effects were different for the two substances. While there was sedation following intraventricular administration of calcium, similar administration of magnesium produced trembling, ataxia and nystagmus. The pigeon fell on to one side and remained in that state for 30 min or more.

Discussion

The results of the present investigation support the concept that the constancy in the ratio between sodium and calcium ions is the inherent mechanism by which the set-point of body temperature is determined. Excess sodium ions shift the set-point to a higher level and excess calcium ions to a lower level. This ionic mechanism, which is common to many species of mammal (Myers, 1974), thus appears to obtain in another class of homoeothermic animals, the birds. As in the mammals, the neurones controlling the set-point can be reached by ions present in solutions injected or infused into a lateral cerebral ventricle of the pigeon.

An increase in the concentration of the calcium ions is highly effective in lowering temperature (Figure 1). On the other hand, a corresponding increase in the concentration of sodium ions is much less effective in raising it. According to Myers (1974) a set-point temperature coupled with the input from the peripheral and central thermoreceptors determines the load error influencing the thermoregulatory response. The present experiments were performed at ambient temperatures of 20–25°C which is within the thermoneutral range for pigeons (King & Farner, 1961). The low magnitude of the sodium hyperthermia and the inability to sustain it cannot, therefore, be attributed to a lack of thermal load error, which would have been the case at high ambient temperature. It is possible that the capacity to attain or maintain a high sodium-calcium ratio in the fluid bathing the set-point neurones is species-dependent. While intense hyperthermia is produced in the unanaesthetized cat during perfusion of a calcium-free saline solution from lateral ventricle to cisterna magna, a weaker effect is obtained in the unanaesthetized rabbit during a similar perfusion (Feldberg & Saxena, 1970a). The present finding demonstrates that the pigeon resembles the rabbit in this respect.

If the pigeon is unable to attain and maintain a high sodium-calcium ratio in the vicinity of the set-point neurones, this might be attributed to the existence of some mechanism which can rapidly and effectively counteract any tendency towards a rise in the relative concentration of sodium ions. Mobilization of calcium ions may be one such mechanism. The presence of such a compensatory mechanism is suggested by the effects of Na_2EDTA. An intraventricular injection of this substance presumably increases the sodium-calcium ratio by chelating calcium ions, resulting in a rise of temperature. This elevation in the sodium-calcium ratio may be rapidly corrected by mobilization of calcium ions and probably overcorrected as the temperature not only returns but falls slightly below the original level (Figure 3a). Such a mechanism once mobilized would appear to be kept effective for a long time so that a second injection of Na_2EDTA might be ineffective in elevating the sodium-calcium ratio and consequently the temperature up to 72 h after the first injection.

By analogy, the ready availability of calcium from extra-circulatory stores has been demonstrated in man by Spencer, Vankinscott, Lewin & Laszlo (1952). The rapid intravenous administration of Na_2EDTA results in hypocalcaemic tetany. However, a slow infusion (less than 15 mg/min) into a normocalcaemic individual elicits no hypocalcaemic symptoms.

$CaNa_2EDTA$ was used essentially as a control for Na_2EDTA. It should not elevate the sodium-calcium ratio and so be without effect on temperature. However, in the present study, it produced similar effects on temperature and behaviour to those obtained with $CaCl_2$. Since $CaNa_2EDTA$ does not dissociate to any significant extent, it seems likely that calcium is acting in its chelated form as certain metals are known to exert their effects on enzymatic processes in this form (Levine, 1970).

Excess of potassium ions in the CSF raised the cloacal temperature but the effect was much weaker than that of sodium ions and an infusion was required to bring about the rise (Figure 4). This finding is in agreement with that of Cooper, Cranston & Honour (1965) who obtained a hyperthermic effect following microinfusion of KCl into the hypothalamus of the rabbit. However, in their experiments isotonic NaCl solution, injected similarly, was without effect on the rectal temperature.

Magnesium resembles calcium in its hypothermic effect but a higher concentration of magnesium was required to produce comparable hypothermia following intraventricular injections. The differences in the time required for the temperature to return to the base line after intraventricular infusion of magnesium and calcium could reflect a difference in the rate at which the two ions are eliminated. The more intense and prolonged effect of magnesium, despite a shorter period of infusion suggests that elimination of magnesium from the CSF is a slower process than that of calcium.

The temperature effects obtained with excess potassium and magnesium in the pigeon were unexpected as neither potassium nor magnesium in concentrations which varied from 2 to 10 times the level in extracellular fluid had any effect on body temperature when these ions were perfused through the cerebral ventricles or microinfused into different regions of the hypothalamus of cat (Myers & Veale, 1971), monkey (Myers *et al.*, 1971), rat (Myers & Brophy, 1971) and golden hamster (Myers & Buckman, 1972). However, the concentrations of these ions employed in the present study are much higher and milligram quantities of magnesium are known to produce a fall and similar amounts of potassium a rise in temperature when injected into the tuberal area of the cat (Hasma, 1930). Alternatively, the CSF-brain barrier permeability to potassium and magnesium ions may be greater in the pigeon than in the mammals and this may account for the more easily obtainable effect of these ions in the pigeon.

References

CHAWLA, NARESH., JOHRI, M.B.L., SAXENA, P.N. & SINGHAL, K.C. (1974). Effects of catecholamines on thermoregulation in pigeons. *Br. J. Pharmac.*, **51**, 497–502.

COOPER, K.E., CRANSTON, W.I. & HONOUR, A.J. (1965). Effects of intraventricular and intrahypothalamic injection of noradrenaline and 5-HT on body temperature in conscious rabbits. *J. Physiol.*, **181**, 852–864.

FELDBERG, W., MYERS, R.D. & VEALE, W.L. (1970). Perfusion from cerebral ventricle to cisterna magna in the unanaesthetized cat. Effect of calcium on body temperature. *J. Physiol.*, **207**, 403–416.

FELDBERG, W. & SAXENA, P.N. (1970a). Effect on body temperature of perfusing calcium-free saline solution from a lateral ventricle to cisterna magna in the unanaesthetized rabbit. *J. Physiol.*, **207**, 52–53P.

FELDBERG, W. & SAXENA, P.N. (1970b). Mechanism of action of pyrogen. *J. Physiol.*, **211**, 245–261.

HASAMA, B. (1930). Pharmakologische und physiologische studien uber die Schweisszentren. *Arch. exp. Path. Pharmak.*, **153**, 291–308. Quoted in Myers & Veale, 1971.

KING, J.R. & FARNER, D.S. (1961). Energy metabolism, thermoregulation and body temperature. In *Biology and Comparative Physiology of Birds*, Vol. II, ed. Marshall, A.J., pp. 215–288, New York & London: Academic Press.

LEVINE, W.G. (1970). Heavy-metal Antagonists. In *The Pharmacological Basis of Therapeutics*, ed. Goodman, L.S. & Gilman, A. pp. 944–957. Fourth Edn. London, Toronto: The MacMillan Co.

MERLIS, J.K. (1940). The effect of changes in the calcium content of the cerebro-spinal fluid on spinal reflex activity in the dog. *Am. J. Physiol.*, **131**, 67–72.

MYERS, R. D. (1974). An integrative model of monoamine and ionic mechanisms in the hypothalamic control of body temperature. In *Temperature Regulation and Drug Action*. Proceedings of a symposium on Temperature Regulation and Drug Action, ed. Lomax, P., Schonbaum, E. & Jacob, J. pp. 32–42, Paris: S. Krager.

MYERS, R.D. & BROPHY, P.D. (1971). Temperature changes in the rat produced by altering the sodium calcium ratio in the cerebral ventricles. *Neuropharmacology*, **11**, 351–361.

MYERS, R.D. & BUCKMAN, J.E. (1972). Deep hypothermia induced in the golden hamster by altering cerebral calcium levels. *Am. J. Physiol.*, **223**, 1313–1318.

MYERS, R.D. & VEALE, W.L. (1971). The role of sodium and calcium ions in the hypothalamus in the control of body temperature of the unanaesthetized cat. *J. Physiol.*, **212**, 411–430.

MYERS, R.D., VEALE, W.L. & YAKSH, T.L. (1971). Changes in body temperature of the unanaesthetized monkey produced by sodium and calcium perfused through cerebral ventricles. *J. Physiol.*, **217**, 381–392.

Br. J. Pharmac. (1976), **56**, 193–199

ELECTROCONVULSIVE SHOCK INCREASES THE BEHAVIOURAL RESPONSES OF RATS TO BRAIN 5-HYDROXYTRYPTAMINE ACCUMULATION AND CENTRAL NERVOUS SYSTEM STIMULANT DRUGS

JANE P.M. EVANS, D.G. GRAHAME-SMITH, A.R. GREEN & ANN F.C TORDOFF
MRC Unit and University Department of Clinical Pharmacology, Radcliffe Infirmary, Oxford OX2 6HE

1 A single electroconvulsive shock (ECS) of 150 V for 1 s increased the concentration of rat brain 5-hydroxyindoleacetic acid (5-HIAA) but did not alter brain 5-hydroxytryptamine (5-HT) or tryptophan concentrations 3 h later.

2 A single ECS decreased 5-HT synthesis 3 h and 6 h later. Synthesis was back to normal after 24 hours. The ECS-treated rats did not show greater hyperactivity produced by the increased brain 5-HT accumulation following administration of L-tryptophan and tranylcypromine at any time up to 24 h later. This suggests that a single electroshock does not alter 5-HT functional activity.

3 Twenty-four hours after the final ECS of a series of 10 shocks given once daily, the rats were given tranylcypromine and L-tryptophan. They displayed greater hyperactivity than control rats not treated with ECS, suggesting that ECS increased 5-HT functional activity. Brain concentrations of 5-HT, 5-HIAA and tryptophan were then unchanged by ECS. 5-HT synthesis and accumulation of 5-HT following tranylcypromine and L-tryptophan were not altered by ECS.

4 The hyperactivity following administration of the 5-HT agonist 5-methoxy *N,N*-dimethyltryptamine was enhanced by repeated (10 day) ECS, suggesting altered post-synaptic responses to 5-HT receptor stimulation.

5 Repeated ECS enhanced locomotor activity following tranylcypromine and L-DOPA. It did not alter brain noradrenaline or dopamine concentrations.

6 The latent period before a pentylenetetrazol-induced convulsion was shortened by repeated ECS.

7 Following repeated ECS there appears to be increased neuronal sensitivity to certain stimuli producing centrally mediated behavioural stimulation. This is discussed in relation to the mechanism by which electroconvulsive therapy (ECT) produces its therapeutic effect.

Introduction

Despite the fact that electroconvulsive therapy (ECT) has been extensively used for many years to treat psychiatric illness, the mechanism of its action is unknown. Reports on the effect of electroconvulsive shock (ECS) on the biogenic amines of rat brain are contradictory both after single and multiple shocks (Essman, 1973).

We have recently developed a behavioural model to study changes in the metabolism and functional activity of 5-hydroxytryptamine (5-HT) in rat brain (Green & Grahame-Smith, 1975a). This has now been used to study the effects of short and long-term ECS on the functional activity of this amine in rat brain. The effects of ECS on brain 5-HT synthesis and metabolism have also been examined.

Methods

Male Sprague-Dawley rats weighing 150–220 g (Anglia Laboratory Animals, Alconbury, Huntingdon) were used in all experiments. They were housed in groups of 3 and given small animal 41B diet and tap water *ad libitum* for the duration of the experiment.

All drugs were dissolved in 0.9% w/v NaCl solution (saline) or suspended in 1% carboxymethylcellulose in saline and given intraperitoneally. Drugs were obtained from the following sources: L-tryptophan, L-DOPA, 5-methoxy-*N,N*-dimethyltryptamine and pentylenetetrazol (Sigma Chemical Company) and tranylcypromine (S.K.&F.).

Electroconvulsive shocks were applied through ear-clip electrodes from an Edison portable ECT unit.

Shocks (50 cycle/s sinusoidal, 150 V for 1 s) were given to rats lightly anaesthetized with halothane. Control rats were anaesthetized but received no electroconvulsive shocks.

Brain 5-hydroxytryptamine (5-HT) and 5-hydroxyinoleacetic acid (5-HIAA) concentrations were measured by the method of Curzon & Green (1970), tryptophan by that of Denckla & Dewey (1967) and dopamine and noradrenaline by the method of Chang (1964). Brain 5-HT synthesis was determined by the method of 5-HT accumulation following monoamine oxidase (MAO) inhibition as described by Neff & Tozer (1968). Activity was measured on groups of 3 animals with Animex activity meters (sensitivity and tuning: 30 µA) as described previously (Grahame-Smith, 1971a; Green & Grahame-Smith, 1974a). Graphs show results of typical experiments performed at least twice.

Results

Effect of a single electroshock on rat brain 5-hydroxytryptamine, 5-hydroxyindoleacetic acid and tryptophan concentrations

Groups of rats were anaesthetized and given either a single shock or no shock. They were killed either 30 min or 180 min later and brain 5-HT and 5-HIAA determined. No change in the concentration of either compound was observed 30 min after treatment. However, 3 h after treatment the ECS group had a higher concentration of 5-HIAA although 5-HT concentrations were unchanged (Table 1).

No evidence was obtained for any alteration of brain tryptophan concentrations following a single ECS (Table 1).

Effect of a single ECS on brain 5-hydroxytryptamine synthesis at various times after the shock

Shields (1972) and Tagliamonte, Tagliamonte, Di Chiara, Gessa & Gessa (1972) found brain 5-HIAA increased following a single ECS but 5-HT synthesis rates were not studied. The rate of 5-HT synthesis at various times after a single electroshock was therefore examined by measuring the rate of 5-HT accumulation following tranylcypromine.

Groups of rats were anaesthetized and given a single electroshock or no shock. One, 3, 6 and 24 h later both groups were given tranylcypromine (20 mg/kg) and the rate of 5-HT accumulation measured 60 min later. Anaesthesia did not alter the rate of 5-HT synthesis at any time as judged by the rate of 5-HT accumulation following tranylcypromine administration to untreated rats (Table 2). However, the rate of 5-HT synthesis was decreased significantly 3 and 6 h after a single electroshock. The rate had returned to normal by 24 h (Table 2).

Effect of ECS on the hyperactivity following tranylcypromine and L-tryptophan administration

To see whether the functional activity of rat brain 5-HT was altered by ECS we examined the effect of a single electroshock on the behavioural response to tranylcypromine and L-tryptophan administration, a procedure which increases 5-HT synthesis and its 'spill-over' into functional activity in the brain (Grahame-Smith, 1971a; Grahame-Smith, 1973). Previously, it has been shown that compounds altering 5-HT compartmentation and release, re-uptake or post-synaptic responses in the brain will cause alterations in the hyperactivity following administration of tranylcypromine and L-tryptophan (for review see Green & Grahame-Smith, 1975a).

Rats were anaesthetized and then given a single ECS or no shock. One, 3, 6 and 24 h later both groups were given tranylcypromine (20 mg/kg) followed 30 min later by L-tryptophan (100 mg/kg) and activity measured. No difference in the hyperactivity response to tranylcypromine and L-tryptophan was observed between the groups at any time.

Effect of ECS given once daily for 10 days on rat brain tryptophan, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid concentrations and the rate of 5-hydroxytryptamine synthesis

Since no behavioural changes were observed after one electroshock the effects of a single ECS given daily for

Table 1 Effect of single electroconvulsive shock (ECS) on rat brain 5-hydroxytryptamine (5-HT) 5-hydroxyindoleacetic acid (5-HIAA) and tryptophan 3 h later

	Brain concentrations in µg/g brain (wet wt)		
Treatment	*5-HT*	*5-HIAA*	*Tryptophan*
Control	0.38 ± 0.01 (14)	0.23 ± 0.01 (14)	2.19 ± 0.19 (5)
ECS	0.38 ± 0.02 (13)	0.27 ± 0.01 (14)*	1.86 ± 0.12 (6)

Results expressed as mean ± s.e. mean. No of observations in brackets
* Different from anaesthetized control $P < 0.01$

10 days was examined. Rats were anaesthetized and given a single shock or no shock at the same time of day (12 h 30 min–13 h 30 min) for 10 days. Twenty-four hours after the final shock both groups were killed and brain tryptophan, 5-HT and 5-HIAA concentrations measured. A further ECS treated group received tranylcypromine (20 mg/kg) and 5-HT accumulation was measured 1 h later. There was no change following repeated ECS in the concentration of 5-HT, 5-HIAA or tryptophan in the brain. Nor was any change observed in the rate of 5-HT synthesis (Table 3).

Effect of repeated ECS on the hyperactivity following tranylcypromine and L-tryptophan

Rats were anaesthetized and given a single shock or no shock for 10 days as described above. Twenty-four hours after the final treatment both groups were given tranylcypromine (20 mg/kg) followed 30 min later by L-tryptophan (50 mg/kg). Activity was measured simultaneously on both groups. Following tranylcypromine the ECS-treated rats showed a small but consistently greater amount of locomotor activity than the control group (approximately 30 movements/min or more). When tryptophan was administered both groups displayed the characteristic syndrome of hyperactivity previously reported following this treatment (Grahame-Smith, 1971a). However, the ECS-treated group was more active than the control and the hyperactivity also started earlier in this group. This was true even when the initially greater activity following tranylcypromine was subtracted from the results (Figure 1).

There was no difference between the groups in the rate of brain 5-HT accumulation 90 min after L-

Table 2 Effect of a single electroconvulsive shock (ECS) on rat brain 5-hydroxytryptamine (5-HT) synthesis at various times after the shock

Treatment	*Time after treatment (h)*	*Brain 5-hydroxytryptamine conc μg 5-HT/g brain (wet wt)* *Saline*	*Tranylcypromine (20 mg/kg)*	*Rate of 5-hydroxytryptamine synthesis (μg g^{-1} h^{-1})*
Untreated	–	0.41 ± 0.03 (6)	0.60 ± 0.03 (4)	0 19
Control	1	0 34 ± 0.06 (5)	0.58 ± 0.02 (3)	0 24
ECS	1	0.38 ± 0 08 (5)	0.59 ± 0.01 (5)	0.21
Control	3	0.38 ± 0.01 (14)	0.56 ± 0.02 (6)	0.18
ECS	3	0.38 ± 0.02 (13)	0.50 ± 0.02 (9)*	0.12
Control	6	0.35 ± 0.04 (3)	0.57 ± 0.03 (6)	0.22
ECS	6	0.37 ± 0.03 (3)	0.49 ± 0 01 (7)*	0.12
Control	24	0.38 ± 0.02 (3)	0.58 ± 0.03 (3)	0.20
ECS	24	0.37 ± 0 02 (3)	0.57 ± 0.02 (4)	0.20

Results are expressed as mean ± s.e. mean with number of observations in brackets. Rate of synthesis calculated by determination of the accumulation of 5-HT 1 h after tranylcypromine (20 mg/kg).
* Different from control, $P < 0.01$.

Table 3 Effect of daily electroconvulsive shock (ECS) for 10 days on the concentration of rat brain tryptophan, 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA) and the rate of 5-HT synthesis 24 h after the final shock

Treatment	*Brain concentrations in μg/g brain (wet wt)* *Tryptophan*	*5-HIAA*	*5-HT*	*5-HT conc 60 min after tranylcypromine (20 mg/kg)*	*Rate of 5-HT synthesis (μg g^{-1} h^{-1})*
Control	1.88 ± 0.07 (5)	0.29 ± 0.01 (5)	0.38 ± 0 04 (5)	0.65 ± 0.03 (6)	0.27
ECS	2.05 ± 0.05 (4)	0.29 ± 0.01 (4)	0.37 ± 0.02 (4)	0.62 ± 0.04 (5)	0.25

Results expressed as mean ± s.e. mean with number of observations in brackets. Rate of 5-HT synthesis determined by measuring rate of 5-HT accumulation 1 h after inhibition of MAO by tranylcypromine (20 mg/kg).

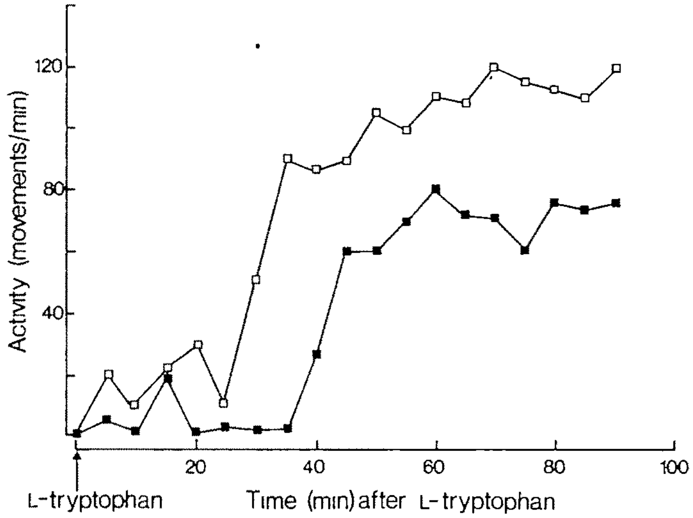


Figure 1 Effect of repeated ECS on the hyperactivity following tranylcypromine and L-tryptophan Rats were given a single ECS each day for 10 days. Twenty-four hours after the final shock they were injected with tranylcypromine (20 mg/kg) and L-tryptophan (50 mg/kg) 30 min later. Figure shows the hyperactivity following the tryptophan in the control (halolane anaesthesia only) group (■) and the ECS group (□). For experimental details see methods section

tryptophan administration (control group: 0.81 ± 0.04 ($n=6$); ECS group: 0.82 ± 0.06 ($n=6$) 90 min after L-tryptophan administration).

Effect of repeated ECS on the hyperactivity following tranylcypromine and 5-methoxy N,N-*dimethyltryptamine*

Since there were no apparent differences in the metabolism of brain 5-HT in the control and experimental groups to explain the enhanced response to 5-HT release it seemed possible that there was a change in the post-synaptic response to the released transmitter. This was tested indirectly with the 5-HT analogue 5-methoxy *N,N*-dimethyltryptamine (5-MeODMT). It has been suggested that this compound directly stimulates post-synaptic 5-HT receptors allowing observation to be made of changes occurring at the receptor site or in the activity of neural systems beyond this involved in producing the complete behavioural response (Grahame-Smith, 1971b).

Rats were given ECS for 10 days as described above. Twenty-four hours after the final shock both groups were injected with tranylcypromine (20 mg/kg) and with 5-MeODMT (2 mg/kg) given 30 min later. The ECS-treated group did not show any increase in the peak activity after this dose of 5-MeODMT but the activity was more prolonged (Figure 2). When a lower dose of 5-MeODMT was used (1 mg/kg) the ECS-treated group showed both a greater peak activity and subsequent enhanced activity compared to the control group. This suggests an increased post-synaptic response to stimulation.

Effect of repeated ECS on the hyperactivity following tranylcypromine and L-DOPA

Previously it has been shown that the hyperactivity resulting from increased 5-HT receptor stimulation is dependent upon adequate brain dopamine concentrations and it appeared that the dopaminergic system involved lies at some point between the 5-HT neurones initiating the response and the total neuronal mechanisms responsible for the expression of the hyperactivity syndrome (Green & Grahame-Smith, 1974a). In order to see whether the presumed increased post-synaptic responses to 5-HT activity after repeated ECS was mediated through changes in this dopaminergic system we investigated the effect of repeated ECS on the locomotor stimulation following tranylcypromine and L-DOPA. This hyperactivity is not identical to that following tranylcypromine and tryptophan. It can nevertheless be measured on activity meters.

Rats were shocked for 10 days as described above. Twenty-four hours after the final shock they were injected with tranylcypromine (20 mg/kg) followed 30 min later by L-DOPA (50 mg/kg).

Rats given ECS showed much more activity than control animals (Figure 3). No evidence was found for

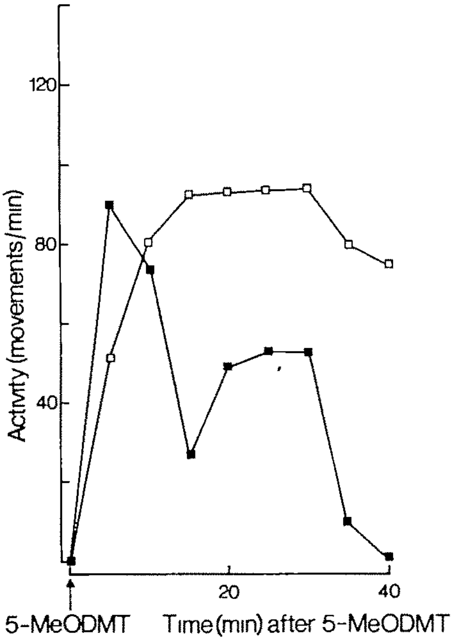


Figure 2 Effect of repeated electroconvulsive shock (ECS) on the hyperactivity following tranylcypromine and 5-methoxy *N,N*-dimethyltryptamine (5-MeODMT). Rats were given a single ECS each day for 10 days. Twenty-four hours after the final shock they were injected with tranylcypromine (20 mg/kg) and with 5-MeODMT (2 mg/kg) 30 min later. Figure shows the hyperactivity following the 5-MeODMT in the control (halothane anaesthesia only) group (■) and the ECS group (□). For experimental details see methods section.

changes in the steady state concentration of brain dopamine or noradrenaline in rats treated with ECS for 10 days.

Effect of a single ECS on the hyperactivity following tranylcypromine and L-DOPA 10 days later

To see whether the enhanced response to tranylcypromine and L-DOPA following repeated electroshock was due to the repeated shocks or because of a changed response present 10 days after the first shock, we gave a single shock to a group of rats on day 1 and challenged them with tranylcypromine (20 mg/kg) and L-DOPA (50 mg/kg) as described above on day 11. No difference in locomotor activity was observed between the control and single ECS-treated groups.

Effect of repeated ECS on the latent period before convulsion following pentylenetetrazol administration

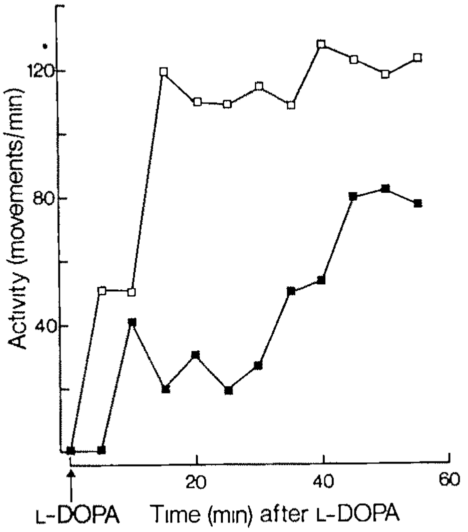


Figure 3 Effect of repeated electroconvulsive shock (ECS) on the hyperactivity following tranylcypromine and L-DOPA Rats were given a single ECS each day for 10 days. Twenty-four hours after the final shock they were injected with tranylcypromine (20 mg/kg) and with L-DOPA (50 mg/kg) 30 min later. Figure shows the hyperactivity following the L-DOPA in the control (halothane anaesthesia only) group (■) and the ECS group (□). For experimental details see methods section.

Because ECS enhanced the behavioural changes due to increases in brain 5-HT and dopamine we decided to see whether it was altering neuronal sensitivity more generally. This was done by examining the effect of repeated ECS on the time taken for rats to convulse following pentylenetetrazol administration.

Rats were shocked for 10 days as described earlier. Twenty-four hours after the last shock they were given pentylenetetrazol (80 mg/kg) and the time taken

Table 4 Effect of electroconvulsive shock (ECS) on length of latent period before pentylenetetrazol-induced convulsions

Treatment	*Time (s) after pentylenetetrazol before first convulsion*
Control	345 ± 152 (5)
ECS	75 ± 16 (5)*

Rats were anaesthetized and given a single ECS or no shock (control) for 10 days. On day 11 both groups were given pentylenetetrazol (80 mg/kg) and the time until the first convulsion measured. Results expressed as mean ± s.e. mean of time taken to convulse Significance calculated using Mann-Whitney non-parametric statistics (Snedecor & Cochran, 1967)

* $P<0.01$.

between injection and convulsion measured. The group treated with ECS convulsed after a shorter latent period than the control animals (Table 4).

Discussion

Both Shields (1972) and Tagliamonte *et al.* (1972) found an increased brain 5-HIAA concentration 3 h after a single ECS. We have confirmed this and like Shields (1972) are unable to find the concomitant rise of brain tryptophan reported by Tagliamonte *et al.* (1972).

Measurement of brain 5-HT turnover, far from showing an increase, indicated clearly that 5-HT synthesis is decreased approximately 50% both 3 h and 6 h after the shock. Since electrical stimulation of brain slices leads to a release of 5-HT from its stores (Chase, Breese & Kopin, 1967), ECS might produce similar changes *in vivo* leading to an increased concentration in the synaptic cleft, following which it is metabolized by MAO producing the transient 5-HIAA increase. This postulated increase in synaptic cleft concentration of the transmitter would be expected to cause an increased stimulation of the receptor (Meek, Fuxe & Andén, 1970; Modigh, 1973a) and a decrease in the rate of 5-HT synthesis (Meek & Werdinius, 1970; Green & Grahame-Smith, 1975b) probably because increased receptor stimulation causes a 'feedback inhibition' of synthesis, possibly via an action on nerve impulse flow (Modigh, 1973b).

The fact that a single ECS had no effect on the hyperactivity response following tranylcypromine and L-tryptophan suggests that one ECS does not affect the functional activity of 5-HT (for review see Green & Grahame-Smith, 1975a). After 10 days of repeated ECS, however, there was considerable enhancement of the hyperactivity following tranylcypromine and tryptophan. Twenty-four hours after the last ECS of 10 daily shocks there was no change in the steady state concentration of brain tryptophan, 5-HT, 5-HIAA or the synthesis rate of 5-HT to account for this change. It seemed possible therefore that the increased hyperactivity was due to an alteration of the post-synaptic response to stimulation by 5-HT. This was confirmed by the observation that ECS enhanced the hyperactivity response to the 5-HT agonist 5-MeODMT which probably stimulates post-synaptic 5-HT receptors (Grahame-Smith, 1971b).

The activity following tranylcypromine and L-DOPA was considerably enhanced by ECS, although ECS did not alter the steady state concentration of dopamine or noradrenaline. This result suggests that ECS alters post-synaptic responses of the dopaminergic systems in the brain. Modigh (1975) also recently reported that repeated but not single ECS to mice leads to enhanced catecholaminergic post-synaptic sensitivity and our results are in accord with this interpretation, although they suggest that the 5-hydroxytryptaminergic system may have been similarly altered. However, in view of the proposed involvement of dopaminergic system in the production of the hyperactivity resultant upon increased brain 5-HT accumulation (Green & Grahame-Smith, 1974a) it is not clear whether this alteration is due solely to changes in the dopaminergic system.

Similar alterations in hyperactivity without observable biochemical changes were seen in a previous study of Thyrotorophin Releasing Hormone (TRH) (Green & Grahame-Smith, 1974b). TRH appears to alter neuronal sensitivity to certain pharmacological stimuli. Both repeated ECS and TRH shortened the latent period before pentylenetetrazol-induced convulsions, suggesting that ECS also alters neuronal sensitivity more generally in the brain than just to the amine neurotransmitters. This striking similarity between the effect of TRH and repeated ECS tempts us to propose a unitary mode of action since it has been previously suggested that certain brain polypeptides or proteins with a short biological half life were involved in mediating or modulating neuronal responses to central monoamine activity (Grahame-Smith, 1972; Green & Grahame-Smith, 1975a). ECS might result in the greater production of these polypeptides or proteins, or in their increased effect. The hypothesis might also explain the effects of ECS on animal behaviour which may be analogous with the therapeutic effects of repeated ECT in man. Tricyclic anti-depressants and monoamine oxidase inhibitors (with or without tryptophan) are thought to act by increasing monoamine concentrations in the synaptic cleft, resulting in greater stimulation of the post-synaptic receptor site. In contrast, ECT appears to be working by increasing the post-synaptic responses to the stimulation of the post-synaptic receptor site.

Please address reprint requests to A.R.G. We thank Smith, Kline and French for generous supplies of tranylcypromine.

References

CHANG, C.C. (1964). A sensitive method for spectrophotofluorometric assay of catecholamines. *Int. J. Neuropharmac.*, **3**, 643–649.

CHASE, T.N., BREESE, G.R. & KOPIN, I.J. (1967). Serotonin release from brain slices by electrical stimulation: regional differences and effect of LSD. *Science*, **157**, 1461–1463.

CURZON, G. & GREEN, A.R. (1970). Rapid method for the determination of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in small regions of rat brain. *Br. J. Pharmac.*, **39**, 653–655.

DENCKLA, W.D. & DEWEY, H.K. (1967). The determination of tryptophan in plasma, liver and urine. *J. lab. clin. Med.*, **69**, 160–169.

ESSMAN, W.B. (1973). *Neurochemistry of cerebral electroshock*. Flushing, New York: Spectrum Publications.

GRAHAME-SMITH, D.G. (1971a). Studies *in vivo* on the relationship between brain tryptophan, brain 5-HT synthesis and hyperactivity in rats treated with a monoamine oxidase inhibitor and L-tryptophan. *J. Neurochem.*, **18**, 1053–1066.

GRAHAME-SMITH, D.G. (1971b). Inhibitory effect of chlorpromazine on the syndrome of hyperactivity produced by L-tryptophan or 5-methoxy-*N,N*-dimethyltryptamine in rats treated with a monoamine oxidase inhibitor. *Br. J. Pharmac.*, **43**, 856–864.

GRAHAME-SMITH, D.G. (1972). The prevention by inhibitors of brain protein synthesis of the hyperactivity and hyperpyrexia produced in rats by monoamine oxidase and the administration of L-tryptophan or 5-methoxy-*N,N*-dimethyltryptamine. *J. Neurochem.*, **19**, 2409–2422.

GRAHAME-SMITH, D.G. (1973). Does the total turnover of brain 5-HT reflect the functional activity of 5-HT in brain. In *Serotonin and Behavior*, ed. Barchas, J. & Usdin, E., pp. 5–7. New York: Academic Press.

GREEN, A.R. & GRAHAME-SMITH, D.G. (1974a). The role of brain dopamine in the hyperactivity syndrome produced by increased 5-hydroxytryptamine synthesis in rats. *Neuropharmacology*, **13**, 949–959.

GREEN, A.R. & GRAHAME-SMITH, D.G. (1974b). TRH potentiates behavioural changes following increased brain 5-hydroxytryptamine accumulation in rats. *Nature, Lond.*, **251**, 524–526.

GREEN, A.R. & GRAHAME-SMITH, D.G. (1975a). The effect of drugs on the processes regulating the functional activity of brain 5-hydroxytryptamine. *Nature, Lond.* (in press).

GREEN, A.R. & GRAHAME-SMITH, D.G (1975b). The effect of diphenylhydantoin on brain 5-hydroxytryptamine metabolism and function. *Neuropharmacology*, **14**, 107–113.

MEEK, J. & WERDINIUS, B. (1970). Hydroxytryptamine turnover decreased by the anti-depressant drug chlorimipramine. *J. Pharm. Pharmac.*, **22**, 141–143.

MEEK, J.L., FUXE, K. & ANDÉN, N-E. (1970). Effects of anti-depressant drugs of the imipramine type on central 5-hydroxytryptamine neurotransmission. *Eur. J. Pharmac.*, **9**, 325–332.

MODIGH, K. (1973a). Effects of chlorimipramine and protryptyline on the hyperactivity induced by 5-hydroxytryptophan after peripheral decarboxylase inhibition in mice. *J. Neural. Trans.*, **34**, 101–109.

MODIGH, K. (1973b). Effect of chlorimipramine on the rate of tryptophan hydroxylation in the intact and transected spiral cord. *J. Pharm. Pharmac.*, **25**, 926–928.

MODIGH, K. (1975). Electroconvulsive shock and postsynaptic catecholamine effects: increased psychomotor stimulant action of apomorphine and clonidine in reserpine pretreated mice by repeated ECS. *J. Neural. Trans.*, **36**, 19–32.

NEFF, N.H. & TOZER, T.N. (1968). *In vivo* measurement of brain serotonin turnover. *Adv. Pharmac.*, **6A**, 97–109.

SNEDECOR, G.W. & COCHRAN, W.G. (1967). *Statistical methods*, 6th ed. Ames, Iowa: Iowa State Univ. Press.

SHIELDS, P.J. (1972). Effects of electroconvulsive shock on the metabolism of 5-hydroxytryptamine in the rat brain. *J. Pharm. Pharmac.*, **24**, 919–920.

TAGLIAMONTE, A., TAGLIAMONTE, P., DI CHIARA, G., GESSA, R. & GESSA, G.L. (1972). Increase in brain tryptophan by electroconvulsive shock in rats. *J. Neurochem.*, **19**, 1509–1512.

(Received June 18, 1975.
Revised September 16, 1975)

Br. J. Pharmac. (1976), **56**, 201–207

EFFECTS OF CHRONIC NICOTINE ADMINISTRATION ON THE DENERVATED RAT ADRENAL MEDULLA

F.J. SEIDLER & T.A. SLOTKIN
Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina 27710, U S A.

1 The effects of chronic nicotine administration (1 or 10 mg/kg, s.c., twice daily) were studied in intact and denervated rat adrenal glands to determine the relative roles of central input and direct actions on catecholamines.

2 Catecholamine depletion was obtained in the intact glands from 1–7 days of treatment with 10 mg/kg, with recovery by 14 days of treatment; catecholamines were not decreased in denervated adrenal glands.

3 Catecholamine depletion was accompanied by a decline in functional storage vesicles (determined by [^{3}H]-adrenaline uptake per gland) in the intact side, while no change was seen in the denervated side; the proportion of newly synthesized vesicles increased markedly during 1–7 days of treatment with 10 mg/kg in the intact side, while a much smaller increase of shorter duration was seen in the denervated adrenal gland.

4 Chronic nicotine administration at either dose level induced tyrosine hydroxylase in both intact and denervated glands, but the increase occurred more slowly in the denervated glands.

5 Dopamine β-hydroxylase levels increased similarly in both sides during treatment with nicotine (10 mg/kg).

6 These studies suggest that although long-term adrenal denervation eliminates the catecholamine depletion caused by chronic administration of nicotine, the mechanisms for induction of catecholamine synthesizing enzymes are still capable of responding to the drug.

Introduction

The actions of nicotine on the adrenal medulla are thought to comprise at least three distinct effects: stimulation via central firing of the splanchnic nerve (Patrick & Kirshner, 1971), direct stimulation of chromaffin cells via nicotinic receptors (Schneider, 1969), and to a lesser extent hormonal stimulation via the adrenal cortex (Rubin & Warner, 1975). The loss of adrenal catecholamines resulting from intense stimulation by nicotine and other drugs (morphine, reserpine, insulin) is accompanied by compensatory induction of the catecholamine synthesizing enzymes, tyrosine hydroxylase and dopamine β-hydroxylase, and by accelerated synthesis of catecholamine storage vesicles (Thoenen, Mueller & Axelrod, 1969; Viveros, Arqueros, Connett & Kirshner, 1969; Slotkin & Seidler, 1975; Anderson & Slotkin, 1975). As a result, chronic treatment of rats with nicotine eventually results in a return of adrenal catecholamine levels to normal despite continued drug administration (Slotkin & Seidler, 1975); the high levels of catecholamine synthesizing enzymes and storage vesicle synthesis can thus replace the amines being lost through accelerated secretion.

It is unclear, however, to what extent adrenal catecholamine depletion and compensatory changes during chronic nicotine administration can be accounted for on the basis of direct effects *versus* neurally-mediated stimulation of the tissue, or whether the two types of stimulation produce different biochemical changes. In the current study, rats with denervated adrenals have been used in order to determine the role of central input in the effects of nicotine, and to evaluate ways in which catecholamine stores are maintained in the absence of normal splanchnic activity.

Methods

Male Sprague-Dawley rats (Zivic-Miller) had their left adrenal glands denervated surgically by splanchnic section 10 days before the start of the experiment, and completeness of denervation established by the absence of a secretory response to insulin (Slotkin & Kirshner, 1973a). Rats were given either 1 or 10 mg/kg nicotine subcutaneously twice daily for two weeks; control rats received the same surgical procedure, but were administered 0.9% w/v NaCl solution (saline). Animals were killed by decapitation at 4 h, 24 h, 7 days and 14 days after initiation of drug administration, and except for the first time point, decapitation occurred 12 h after the last nicotine injection.

Individual glands were removed, cleaned of fat and homogenized (glass-to-glass) in 2.5 ml of 0.3 M sucrose containing 0.025 M Tris (pH 7.4) and 10^{-5} M iproniazid; 0.1 ml was removed and deproteinized with 1.9 ml of 3.5% perchloric acid and centrifuged for 10 min at 26,000 *g*. The supernatant was analyzed for catecholamines by the trihydroxyindole method using an autoanalyzer (Merrills, 1963). Duplicate 0.2 ml portions of the homogenate were added to 0.2 ml of water containing 400 u of beef catalase (Sigma) and assayed for dopamine β-hydroxylase (Friedman & Kaufman, 1965), using 10 µM [^{3}H]-tyramine as substrate; para-hydroxymercuribenzoate (optimal concentration, 0.5 mM) was used to inactivate endogenous inhibitors.

The remainder of the homogenate was centrifuged at 800 *g* for 10 min and 1 ml of the supernatant used for the determination of adrenaline uptake (*vide infra*). The rest of the 800 *g* supernatant was centrifuged at 26,000 *g* for 10 min to sediment the catecholamine storage vesicles and the supernatant used for duplicate determinations of tyrosine hydroxylase activity by the method of Waymire, Bjur & Weiner (1971), using 100 µM [^{14}C]-tyrosine as substrate.

The abilities of the storage vesicles to incorporate [^{3}H]-adrenaline were determined by standard techniques as described previously (Slotkin & Kirshner, 1973a). For each adrenal preparation, duplicate tubes were prepared containing 0.5 ml of the 800 *g* supernatant, 5 µmol of ATP and Mg^{2+}, 5 µCi of [^{3}H]-adrenaline, 0.1 µmol of unlabelled adrenaline and sucrose-Tris in a final volume of 1 ml. Samples were incubated at 30°C for 30 min while duplicates were kept on ice to serve as blanks. Uptakes were stopped by the addition of 2 ml of ice-cold sucrose-Tris and the labelled vesicles sedimented at 26,000 *g* for 10 minutes. The supernatant was deproteinized with an equal volume of 7% perchloric acid, centrifuged at 26,000 *g* for 10 min and assayed for catecholamines and radioactivity; this enabled determination of the specific activity of the labelling medium. The vesicular pellet was washed with fresh sucrose-Tris and recentrifuged twice, and then suspended in 3 ml of 3.5% perchloric acid, centrifuged and the supernatant analyzed for catecholamines and radioactivity. Although contaminating particles are present, under these conditions labelling occurs solely in the storage vesicles (Slotkin & Kirshner, 1971, 1973b). The temperature-dependent uptake was calculated as described previously (Slotkin & Kirshner, 1973a) and expressed as uptake per gland (a composite measure reflecting the number of functional vesicles as well as the uptake capability of each vesicle) and as uptake per unit of catecholamines (a measure of the abilities of individual vesicles to incorporate [^{3}H]-adrenaline relative to endogenous content, independently of the number of vesicles present). Nicotine *in vitro* has no direct effect on uptake (Carlsson, Hillarp & Waldeck, 1963).

In order to compare the effects of nicotine after surgical denervation with those after nicotinic receptor blockade, other experiments were done in which rats were pretreated with a blocking agent (chlorisondamine, 7.5 mg/kg s.c.) 30 min prior to receiving a single injection of nicotine (1 or 10 mg/kg s.c.). Three and 24 h after the second injection, animals were killed and the adrenals were homogenized in 2 ml of 0.15 M KCl. Aliquots (0.1 ml) were withdrawn for catecholamine analysis and the remaining homogenate either centrifuged at 26,000 *g* and the supernatant used for tyrosine hydroxylase determinations, or else diluted with an equal volume of 10 mM Tris (pH 7.2), centrifuged and the supernatant used for assay of ornithine decarboxylase according to Anderson & Schanberg (1975), with 12 µM [^{14}C]-ornithine as substrate.

Results are expressed as mean ± s.e., and levels of significance calculated by Student's *t*-test (Wine, 1964).

(±)-Adrenaline-[7-^{3}H], tyramine-[G-^{3}H], L-tyrosine-[1-^{14}C] and DL-ornithine-[1-^{14}C] were purchased from New England Nuclear Corp. (−)-Adrenaline bitartrate and nicotine were obtained from Winthrop Laboratories and Sigma Chemical Corp., respectively, and chlorisondamine from Ciba Pharmaceuticals.

Results

Administration of low doses of nicotine (1 mg/kg) had little effect on catecholamine levels in either innervated or denervated adrenal glands (Figure 1). With the higher dose (10 mg/kg) however, catecholamines in the intact glands were markedly depleted 4 to 24 h after beginning drug administration, while in the denervated side there was no change. Catecholamines in the innervated glands had returned to control levels by day 14 despite continued drug administration; at no time were the levels in the denervated glands significantly different from those of denervated controls.

The effects of nicotine on adrenal tyrosine hydroxylase activity are shown in Figure 2. After nicotine (1 mg/kg), tyrosine hydroxylase was elevated at 24 h in the intact gland while there was no significant increase in the denervated side until 7 days. With the high dose of nicotine, tyrosine hydroxylase activities in both sides were elevated by 24 h and remained so for the entire course of the study,

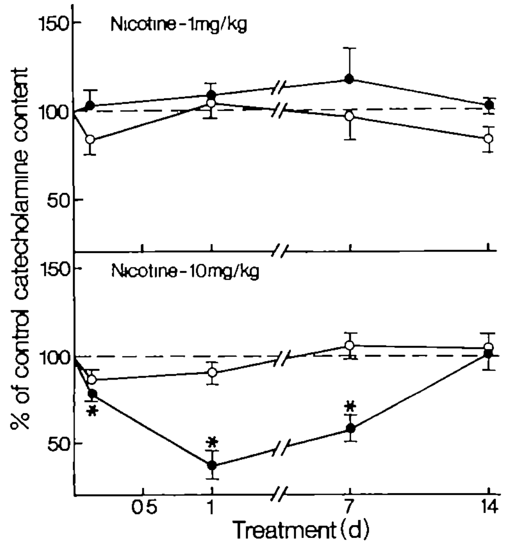


Figure 1 Effects of nicotine (1 mg/kg or 10 mg/kg twice daily) on catecholamine content in innervated (●) and denervated (O) rat adrenal glands Points and bars represent means ± s.e. of 5 animals expressed as percentage of control catecholamine content; * denotes significant differences from control ($P<0.05$) Control values were 8.74±0.46 μg/gland in the innervated side, and 7.66±0.46 μg/gland in the denervated side (20 animals).

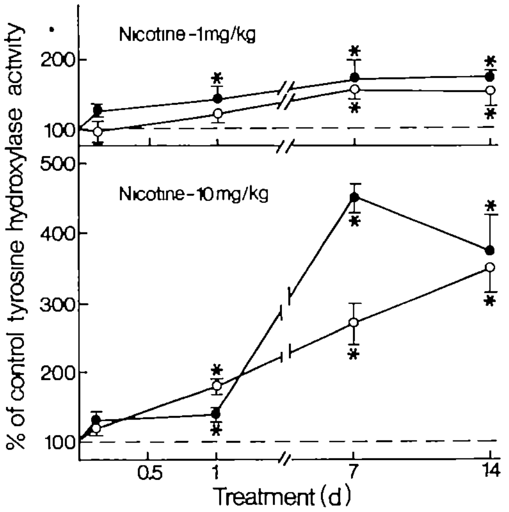


Figure 2 Effects of nicotine (1 mg/kg or 10 mg/kg twice daily) on tyrosine hydroxylase activity in innervated (●) and denervated (O) rat adrenals Points and bars represent means ± s.e. of 5 animals expressed as percentage of control; * denotes significant differences from control ($P<0.05$). Control values were 12.3±0.8 nmol $^{14}CO_2$ evolved/h per gland in the innervated side, and 10.9±0.3 nmol/h per gland in the denervated side (20 animals).

reaching levels as high as 4 times control in the intact glands and 3 times control in the denervated glands; the denervated glands required a longer period of treatment to reach the maximum level.

Adrenal dopamine β-hydroxylase activity in intact glands showed only minor changes during administration of the low dose of nicotine (Figure 3). However at the high dose, dopamine β-hydroxylase was elevated in both sides after 7 days, reaching levels twice to three times control.

To determine whether nicotine-induced changes in dopamine β-hydroxylase reflected alterations in storage vesicle synthesis, the abilities of isolated vesicles to incorporate [^{3}H]-adrenaline were evaluated. Uptake per gland remained approximately at control levels in both sides with the low dose of nicotine (Table 1). However at the high dose, uptake per gland was markedly reduced in the intact side between 1 and 7 days of drug administration, while in the denervated side there was little change.

Uptake per 100 μg of endogenous catecholamine showed little or no consistent change in either side with the low dose of nicotine, but with the high dose of nicotine at 24 h there was a marked enhancement in the innervated side with a smaller increase in the denervated side (Table 1). Uptake per 100 μg of

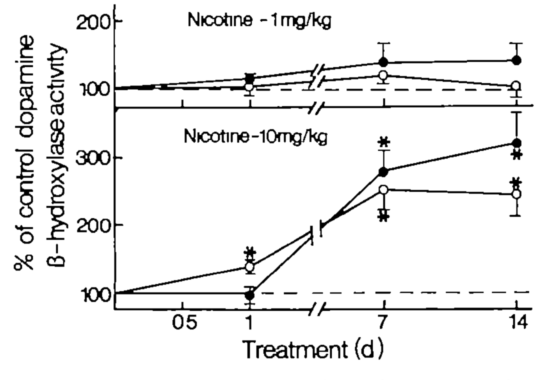


Figure 3 Effects of nicotine (1 mg/kg or 10 mg/kg twice daily) on dopamine β-hydroxylase activity in innervated (●) and denervated (O) rat adrenals. Points and bars represent means ± s e. of 5 animals expressed as percentage of control; * denotes significant differences from control ($P<0.05$ or better). Control values were 0.681±0.037 nmol [^{3}H]-octopamine formed/h per gland in the innervated side, and 0.520±0.050 nmol formed/h per gland in the denervated side (20 animals).

Table 1 Effects of nicotine on uptake of [^{3}H]-adrenaline into isolated storage vesicles from intact and denervated rat adrenals

Nicotine	Time after start of treatment	[^{3}H]-adrenaline uptake per gland		[^{3}H]-adrenaline uptake per 100 µg endogenous catecholamines	
		innervated	*denervated*	*innervated*	*denervated*
1 mg/kg	4 h	108 ± 15	99 ± 16	105 ± 8	124 ± 12
	24 h	126 ± 9*	106 ± 15	115 ± 6	98 ± 9
	7 d	101 ± 9	97 ± 14	90 ± 7	98 ± 6
	14 d	102 ± 2	87 ± 4	101 ± 6	107 ± 6
10 mg/kg	4 h	82 ± 5	95 ± 6	103 ± 4	111 ± 9
	24 h	67 ± 9**	120 ± 7	199 ± 13****	132 ± 9***
	7 d	73 ± 7**	109 ± 6	128 ± 9**	101 ± 6
	14 d	84 ± 11	83 ± 7	112 ± 8	80 ± 4***

Nicotine was given subcutaneously twice daily at the dosages indicated. Data represent means ± s.e. of 5 rats per group, as percentages of control. Control values were: innervated gland, 1.96 ± 0.16 nmol/gland, 22.1 ± 1.1 nmol/100 µg of endogenous catecholamines, denervated gland, 1.56 ± 0.15 nmol/gland, 20.0 ± 1.0 nmol/100 µg catecholamines (20 animals).

Significant differences from control are indicated by *($P<0.05$), **($P<0.02$), ***($P<0.005$) or ****($P<0.001$)

Table 2 Effects of chlorisondamine and nicotine on rat adrenal catecholamines, ornithine decarboxylase and tyrosine hydroxylase activities.

First injection	Second injection (30 min later)	3 h after second injection		24 h after second injection	n
		Catecholamine (µg/gland)	*Ornithine decarboxylase (nmol $^{14}CO_2$/h per gland)*	*Tyrosine hydroxylase (nmol $^{14}CO_2$/h per gland)*	
Saline	*Saline*	10.2 ± 0.5	0.00 ± 0.09	5.95 ± 0.34	12
Chlorisondamine (2.5 mg/kg)	*Saline*	10.2 ± 0.3	0.60 ± 0.12****	6.77 ± 0.63	6
Chlorisondamine (7.5 mg/kg)	*Saline*	9.48 ± 0.39	2.85 ± 0.72****	7.31 ± 0.40**	12
Chlorisondamine (20 mg/kg)	*Saline*	9.01 ± 0.82	2.18 ± 0.65***	8.84 ± 0.21****	6
Saline	*Nicotine* (1 mg/kg)	9.39 ± 0.72	0.54 ± 0.12***	7.54 ± 0.63*	6
Saline	*Nicotine* (10 mg/kg)	7.31 ± 0.37****	7.09 ± 1.16****	10.7 ± 0.7****	6
Chlorisondamine (7.5 mg/kg)	*Nicotine* (1 mg/kg)	9.27 ± 0.78	0.53 ± 0.14**	8.79 ± 0.90**	6
Chlorisondamine (7.5 mg/kg)	*Nicotine* (10 mg/kg)	8.95 ± 0.47	3.72 ± 0.75****¶	7.45 ± 0.40**§	6

All drugs were administered s.c. Data represent means ± s.e.

*$P<0.05$ vs. saline–saline; **$P<0.02$, ***$P<0.005$; ****$P<0.001$. ¶$P<0.05$ vs. nicotine alone, not significant vs. chlorisondamine alone. §$P<0.005$ vs. nicotine alone, not significant vs. chlorisondamine alone. n = number of determinations.

catecholamines in the denervated adrenal had declined to normal by 7 days after initiation of the high dose and to slightly below normal by 14 days, while the intact gland remained elevated until 14 days.

The effects of a nicotinic blocking agent on the actions of nicotine are shown in Table 2. In these studies, the activity of ornithine decarboxylase, an enzyme involved in polyamine synthesis, was used as a marker for stimulation of the adrenal gland (Byus & Russell, 1974); the 3 h time point was chosen because in that period, stimulation of the adrenal gland produces an increment in ornithine decarboxylase activity primarily in the medulla as opposed to the cortex (Byus & Russell, 1974). Administration of nicotine (10 mg/kg) alone caused acute catecholamine depletion and marked elevations in ornithine decarboxylase (3 h) and subsequently in tyrosine hydroxylase (24 h). Pretreatment with the blocking agent, chlorisondamine, eliminated the nicotine-induced amine depletion and reduced ornithine decarboxylase and tyrosine hydroxylase activities to those seen after chlorisondamine alone. The partial stimulatory effect of chlorisondamine itself is consistent with previous observations that a number of ganglionic blocking agents also evoke stimulation of the adrenal medulla (Mueller, Thoenen & Axelrod, 1970a; Carlsson & Lindqvist, 1974; Slotkin, Seidler, Lau, Bartolomé & Schanberg, 1976).

Discussion

Agents which either stimulate nicotinic receptors directly or which evoke central stimulation of the sympatho-adrenal axis, cause quantal exocytotic release of adrenomedullary catecholamines (Schneider, 1969; Viveros *et al.*, 1969; Slotkin & Kirshner, 1973a; Anderson & Slotkin, 1975; Slotkin & Seidler, 1975) and a parallel decline in amine levels and in the number of functional adrenal storage vesicles (Viveros *et al.*, 1969; Slotkin & Kirshner, 1973a). In the present study, repeated administration of nicotine (10 mg/kg) produced, as expected, a depletion of catecholamines and also a reduction in the uptake per gland of [^{3}H]-adrenaline, which illustrates the reduced number of functional vesicles. However, in denervated adrenal glands in the same rats, no catecholamine depletion was observed and there was no decrease in vesicle function, indicating that these actions of nicotine require an intact nerve supply.

Three hypotheses could explain this phenomenon: first, nicotine could act only reflexly via the splanchnic nerve and not directly on the adrenal gland; this is unlikely, since direct nicotine-induced secretion of catecholamines already has been demonstrated (Schneider, 1969; Tsujimoto & Nishikawa, 1974). Second, long-term denervation could alter the number and/or function of nicotinic receptors in the adrenal gland such that stimulation by nicotine can no longer occur; this is also unlikely, since nicotine was able to increase activities of both tyrosine hydroxylase and dopamine β-hydroxylase, indicating adrenomedullary stimulation by the drug (Thoenen, 1974). Third, denervation could alter the processes participating in stimulus-secretion coupling, such that nicotine produces a smaller secretory response; since there is substantial evidence that denervation can indeed change the secretory response to a number of other drugs, this may explain the inability of nicotine to cause significant catecholamine depletion. For example, acute (short-term) surgical or chemical interruption of neural input to the adrenal medulla prevents the immediate secretory effects of drugs which act via splanchnic stimulation (Stewart & Rogoff, 1916; Mueller *et al.*, 1970a; Viveros, Arqueros & Kirshner, 1971; Yoshizaki, 1973) but upon chronic denervation (or in neonatal rats, which do not have a functional nerve supply to the adrenal gland) the secretory response reappears (Yoshizaki, 1973; Anderson & Slotkin, 1976; Bartolomé & Slotkin, 1976); however, under those circumstances, the restored secretory response is different from the normal effect in that it probably does not involve nicotinic receptors (Bartolomé & Slotkin, 1976).

The effects of chronic nicotine administration on tyrosine hydroxylase and dopamine β-hydroxylase activities also were different in denervated and in innervated glands. While either low or high doses of nicotine ultimately produced nearly equivalent induction of tyrosine hydroxylase in both types of gland, the increases in the denervated side lagged behind those in the innervated side, indicating that under normal circumstances there are both direct and neural contributions to nicotine-evoked tyrosine hydroxylase induction. In contrast, dopamine β-hydroxylase induction in denervated adrenals exhibited the same time course as in innervated glands, suggesting that the neural component may be relatively less essential in mediating induction of that enzyme. These results are consistent with previous findings in denervated adrenals that other agents also are able to induce dopamine β-hydroxylase fully but are much less effective for tyrosine hydroxylase (Patrick & Kirshner, 1971; Slotkin *et al.*, 1976; Anderson & Slotkin, 1976).

In innervated adrenal glands, the increase in tyrosine hydroxylase activity and replacement of storage vesicles are thought to be determining factors in maintaining catecholamine stores in the presence of increased stimulation; as a consequence of tyrosine hydroxylase induction and new vesicle formation, amine levels can return to normal despite continued drug-induced stimulation. One index of these changes is the uptake per unit (100 µg) of catecholamines, a measure of new vesicle formation; since new vesicles initially have abnormally low catecholamine levels, this index tends to rise if exocytotic loss of amines and intact vesicles exceeds the rate of replacement and to fall when resynthesis approximates loss (Slotkin & Kirshner, 1973a, 1973b; Anderson & Slotkin, 1975; Slotkin & Seidler, 1975).

In the present study, the innervated glands showed an initial (24 h to 7 days) rise in uptake per unit of catecholamine, corresponding to accelerated vesicle turnover and a consequent increase in the number of immature vesicles. Despite continued drug administration, uptake per unit of catecholamine returned to normal in the innervated gland, indicating that amine and vesicle replacement were able to overcome the nicotine-induced increase in secretion. In contrast, the denervated side showed a much smaller initial increase in uptake per unit of catecholamine, supporting the hypothesis that secretion is reduced by denervation. Uptake per unit of catecholamine in denervated glands returned to normal earlier (7 days) than in the intact adrenals despite the smaller degree of tyrosine hydroxylase induction in the denervated side; this is not surprising, since the lower degree of secretion would result in less demand for replacement.

It is unclear whether the effects of chronic nicotine administration on denervated adrenals result purely from direct effects on the tissue or whether secondary actions via changes in adrenal steroids could play a role (Weinshilboum & Axelrod, 1970; Gewirtz, Kvetňanský, Weise & Kopin, 1971; Kvetňanský, 1974); nicotine causes ACTH release from the pituitary as well as evoking direct secretion of adrenocortical steroids (Kershbaum, Pappajohn, Bellet, Hirabayashi & Shafiha, 1968; Suzuki, Ikeda & Narita, 1973; Rubin & Warner, 1975). Since it is unlikely that cutting the splanchnic nerve alters nicotine-induced ACTH release, hormonal effects alone cannot account for the loss of the catecholamine-depleting effect of nicotine in denervated adrenals. The increases seen in tyrosine hydroxylase and dopamine β-hydroxylase activities after chronic nicotine are much higher and occur much sooner than those which can be achieved after large doses of ACTH or glucocorticoids (Weinshilboum & Axelrod, 1970; Mueller *et al.*, 1970b); however, a partial contribution of the pituitary-adrenal axis cannot be ruled out. Nicotine-induced adrenal stimulation as determined by catecholamine secretion, ornithine decarboxylase and tyrosine hydroxylase activities, was blocked completely by administration of chlorisondamine, suggesting that the non-neural component represents effects mediated via nicotinic receptors in the adrenal medulla itself and/or nicotinic receptors involved in ACTH secretion.

In conclusion, the effects of nicotine on regulation of catecholamine and storage vesicle secretion and synthesis reflect several sites of action, and long-term denervation produces alterations in the abilities of nicotine to deplete adrenal catecholamines and to induce catecholamine biosynthetic enzymes.

This work was supported by a grant-in-aid from the American Heart Association and by USPHS DA-00465. Dr Slotkin is recipient of Research Scientist Development Award no. K01–DA–00006 from the National Institute on Drug Abuse.

References

ANDERSON, T.R. & SCHANBERG, S.M. (1975). Effects of thyroxine and cortisol on brain ornithine decarboxylase activity and swimming behavior in developing rat. *Biochem. Pharmac.*, **24**, 495–501.

ANDERSON, T.R. & SLOTKIN, T.A. (1975). Effects of morphine on the rat adrenal medulla. *Biochem. Pharmac.*, **24**, 671–679.

ANDERSON, T.R. & SLOTKIN, T.A. (1976). The role of neural input in the effects of morphine on the rat adrenal medulla. *Biochem. Pharmac.* (in press).

BARTOLOMÉ, J. & SLOTKIN, T.A. (1976). Effects of postnatal reserpine on sympatho-adrenal development in the rat. *Biochem. Pharmac.* (in press).

BYUS, C.V. & RUSSELL, D.H. (1974). Effects of methyl xanthine derivatives on cyclic AMP levels and ornithine decarboxylase activity of rat tissues. *Life Sci.*, **15**, 1991–1997.

CARLSSON, A., HILLARP, N.-Å. & WALDECK, B. (1963). Analysis of the Mg^{++}-ATP dependent storage mechanism in the amine granules of the adrenal medulla. *Acta physiol. scand.*, **59**, suppl. 215, 1–38.

CARLSSON, A. & LINDQVIST, M. (1974). Studies on the neurogenic short-term control of adrenomedullary hormone synthesis. *J. Neur. Trans.* **35**, 181–196.

FRIEDMAN, S. & KAUFMAN, S. (1965). 3,4-dihydroxyphenylethylamine-β-hydroxylase, physical properties, copper content, and role of copper in the catalytic activity. *J. biol. Chem.*, **240**, 4763–4773.

GEWIRTZ, G.P., KVETŇANSKÝ, R., WEISE, V.K. & KOPIN, I.J. (1971). Effects of hypophysectomy on adrenal dopamine β-hydroxylase activity in the rat. *Mol. Pharmac.*, **7**, 163–168.

KERSHBAUM, A., PAPPAJOHN, D.J., BELLET, S., HIRABAYASHI, M. & SHAFIHA, H. (1968). Effect of smoking and nicotine on adrenocortical secretion. *J. Am. Med. Ass.*, **203**, 275–278.

KVETŇANSKÝ, R. (1974). Transsynaptic and humoral regulation of adrenal catecholamine synthesis in stress. *Biochem. Pharmac.*, **23**, suppl. 1, 160–166.

MERRILLS, R.J. (1963). A semiautomatic method for determination of catecholamines. *Analyt. Biochem.*, **6**, 272–282.

MUELLER, R.A., THOENEN, H. & AXELROD, J. (1970a). Inhibition of neuronally induced tyrosine hydroxylase by nicotinic receptor blockade. *Eur. J. Pharmac.*, **10**, 51–56.

MUELLER, R.A., THOENEN, H. & AXELROD, J. (1970b). Effect of pituitary and ACTH on maintenance of basal tyrosine hydroxylase activity in the rat adrenal gland. *Endocrinology*, **86**, 751–755.

PATRICK, R.L. & KIRSHNER, N (1971). Effect of stimulation on the levels of tyrosine hydroxylase, dopamine β-hydroxylase and catecholamines in intact and denervated rat adrenal glands. *Mol. Pharmac.*, **7**, 87–96.

RUBIN, R.P & WARNER, W. (1975). Nicotine-induced stimulation of steroidogenesis in adrenocortical cells of the cat. *Br. J. Pharmac.*, **53**, 357–362.

SCHNEIDER, F.H. (1969). Secretion from the cortex-free bovine adrenal medulla. *Br. J. Pharmac.*, **37**, 371–379.

SLOTKIN, T.A. & KIRSHNER, N. (1971). Uptake, storage and distribution of amines in bovine adrenal medullary vesicles. *Mol. Pharmac.*, **7**, 581–592.

SLOTKIN, T.A. & KIRSHNER, N. (1973a). All-or-none secretion of adrenal medullary storage vesicle contents in the rat. *Biochem. Pharmac.*, **22**, 205–219.

SLOTKIN, T A. & KIRSHNER, N (1973b). Recovery of rat adrenal amine stores after insulin administration. *Mol. Pharmac.*, **9**, 105–116.

SLOTKIN, T.A. & SEIDLER, F.J. (1975). Acute and chronic effects of nicotine on synthesis and storage of catecholamines in the rat adrenal medulla. *Life Sci.*, **16**, 1613–1622.

SLOTKIN, T.A., SEIDLER, F J., LAU, C., BARTOLOMÉ, J. & SCHANBERG, S.M. (1976). Effects of chronic chlorisondamine administration on the sympatho-adrenal axis. *Biochem. Pharmac.* (in press).

STEWART, G.N. & ROGOFF, J.M. (1916). The influence of certain factors, especially emotional disturbances, on the epinephrine content of the adrenals. *J. exp. Med.*, **24**, 709–738.

SUZUKI, T., IKEDA, H. & NARITA, S. (1973). Adrenal cortical secretion in response to nicotine in conscious and anesthetized dogs. *Quart. J. exp. Physiol.*, **58**, 139–142.

THOENEN, H. (1974). Transsynaptic enzyme induction. *Life Sci.*, **14**, 223–235.

THOENEN, H., MUELLER, R.A. & AXELROD, J. (1969). Transsynaptic induction of adrenal tyrosine hydroxylase. *J. Pharmac. exp. Ther.*, **169**, 249–254.

TSUJIMOTO, A. & NISHIKAWA, T. (1974). Comparison of the effects of nicotine in catecholamine release from isolated adrenal glands of dogs and monkeys. *Eur. J. Pharmac.*, **29**, 316–319.

VIVEROS, O.H., ARQUEROS, L., CONNETT, R.J. & KIRSHNER, N. (1969). Mechanism of secretion from the adrenal medulla IV. The fate of the storage vesicles following insulin and reserpine administration. *Mol. Pharmac.*, **5**, 69–82.

VIVEROS, O H., ARQUEROS, L. & KIRSHNER, N. (1971). Mechanism of secretion from the adrenal medulla VII. Effect of insulin administration on the buoyant density, dopamine β-hydroxylase, and catecholamine content of adrenal storage vesicles. *Mol. Pharmac.*, **7**, 444–454.

WAYMIRE, J.C , BJUR, R. & WEINER, N (1971). Assay of tyrosine hydroxylase by coupled decarboxylation of dopa formed from 1-^{14}C-L-tyrosine. *Anal. Biochem.*, **43**, 588–600.

WEINSHILBOUM, R & AXELROD, J. (1970). Dopamine β-hydroxylase activity in the rat after hypophysectomy. *Endocrinology*, **87**, 894–899.

WINE, R.L. (1964). *Statistics for Scientists and Engineers.* Englewood Cliffs, N.J.: Prentice-Hall.

YOSHIZAKI, T (1973). Effects of histamine, bradykinin and morphine on adrenaline release from rat adrenal gland. *Jap. J. Pharmac.*, **23**, 695–699.

*(Received August 8, 1975.
Revised October 6, 1975.)*

Br. J. Pharmac. (1976), **56**, 209–218

SHAPE CHANGE AND AGGREGATION OF BLOOD PLATELETS: INTERACTION BETWEEN THE EFFECTS OF ADENOSINE DIPHOSPHATE, 5-HYDROXYTRYPTAMINE AND ADRENALINE

F. MICHAL[1] & MINA MOTAMED[2]
Department of Pharmacology, University Medical School,
Hills Road, Cambridge CB2 2QD

1 The interaction of effects between 5-hydroxytryptamine (5-HT) and adenosine diphosphate (ADP) on human or rabbit platelets was investigated *in vitro*. The initial platelet shape change and their aggregation were measured in stirred, citrated platelet-rich plasma (PRP) at 37°C by recording the rate and extent of changes in light scattering and light transmission.

2 Both the velocity and extent of aggregation and the velocity and extent of the rapid morphological change caused by ADP were enhanced by simultaneous addition of 5-HT. Methysergide but not imipramine inhibited the 5-HT effects.

3 Platelets were made refractory to the aggregating and shape changing effect of either ADP or 5-HT by repeated aggregation with the particular agent; platelets made refractory to ADP retained their responsiveness to 5-HT and platelets made refractory to 5-HT responded to ADP. Platelets pre-incubated for 3–10 min with 5-HT without aggregation showed greatly reduced aggregation on subsequent addition of ADP. Methysergide inhibited all the effects of 5-HT whilst imipramine was inactive.

4 When the shape change or aggregation of platelets induced by ADP was submaximal, addition of 5-HT increased it further. Pre-incubation of PRP with 5-HT before the addition of ADP resulted in failure of the secondary induction of aggregation or shape change by 5-HT. The secondary induction by 5-HT also did not occur in the presence of methysergide; imipramine had no inhibitory effect. Similar secondary induction of aggregation was shown by adrenaline injected during aggregation by ADP; the adrenaline effect was removed by phentolamine but not by propranolol.

5 Our results show that the initial change in shape of platelets and their aggregation can be induced by ADP or 5-HT in specific manner. The interaction of the effect of these substances on platelets can result in either increase in platelet sensitivity or, under certain conditions, decrease in platelet responsiveness. The increase or depression of platelet reactivity appears to be a highly specific effect and is probably mediated at specific receptors involved with platelet activation.

Introduction

Mammalian blood platelets contain relatively high amounts of 5-hydroxytryptamine (Rand & Reid, 1951) which they are able to concentrate against concentration gradients by a membrane transport system that is coupled to sodium influx and which may be energy-dependent (Hardisty & Stacey, 1955; Born & Gillson, 1959; Sneddon, 1973). It was thought that platelets do not synthetize 5-hydroxytryptamine (Paasonen, 1965) but recently 5-hydroxytryptophan decarboxylase activity has been demonstrated *in vitro* in extracts of human and beef platelets (Marmaras & Mimikos, 1971). Another mechanism is thought to be responsible for the accumulation of 5-hydroxytryptamine (5-HT) in cytoplasmic organelles; specifically that 5-HT forms a stable complex with adenosine triphosphate (Born, Ingram & Stacey, 1958; Da Prada & Pletscher, 1968; Pletscher, Da Prada & Tranzer, 1969). Most of this stored 5-HT is released together with adenosine triphosphate (ATP), by aggregating agents such as thrombin (Grette, 1962), adenosine diphosphate (ADP) and collagen (Packham, Guccione, Chang & Mustard, 1973). It has been suggested that such a release reaction accompanies aggregation, at least *in vitro* (Mills & Roberts, 1967; Mustard & Packham, 1970).

In the platelets of some mammalian species 5-HT induces a rapid morphological change that is followed by reversible aggregation. In human platelet-rich

---

[1 & 2] Present address: Department of Experimental Medicine and Pharmacology, University of Teheran, Shahreza Avenue, Teheran, Iran.

plasma (PRP) aggregation velocity accelerates with 5-HT concentrations up to approximately 10 µM and decelerates again with concentrations above this level (Baumgartner & Born, 1968). In citrated rabbit PRP 5-HT does not itself cause aggregation but potentiates aggregation induced by ADP (Sinakos & Caen, 1967). Furthermore, incubation of platelets with 5-HT can, under certain conditions, diminish the aggregating effect of ADP as well as aggregation by 5-HT itself (Baumgartner & Born, 1969). Experiments with 5-HT antagonists have established that 5-HT initiates aggregation by reacting with a receptor similar to the D-receptor of smooth muscle (Michal, 1969). More recent evidence suggests the existence of two different receptors, one mediating shape change and aggregation by 5-HT and the other uptake of the amine (Born, Juengjaroen & Michal, 1972).

This paper provides additional observations on the shape change and aggregation of platelets induced by 5-HT, and on its interaction with ADP and adrenaline. The effects of the 5-HT antagonists methysergide and imipramine, which block shape change/aggregation by 5-HT and its uptake respectively, are also presented.

Methods

Preparation of platelet-rich plasma

Platelet-rich plasma (PRP) was prepared from human or rabbit citrated blood (trisodium citrate 0.38% final concentration) by centrifugation at 200 *g* for 10 or 15 min respectively. Decanted PRP was kept at room temperature and used as soon as possible after bleeding.

Platelet shape change and aggregation

Photometric records of platelet aggregation and the preceding shape change were obtained by the method of Michal & Born (1971) which allows simultaneous recordings of light transmission and scattering in the PRP at 37°C. When shape change only was required, 10 µl of 0.4M ethylene glycolbis-(2-aminoethyl) tetra-acetic acid was added to each ml of PRP before challenge with agonists. Platelet aggregation and shape change were quantitated by measuring the velocity and extent of the photometric records. When the effect produced by one agent was enhanced or inhibited in the presence of another agent, the responses obtained were expressed as percentage of the normal controls. When measuring the influence that pre-incubation with 5-HT had on the subsequent aggregation or shape change, a strict timing schedule was observed so that the total period during which the samples were warmed and stirred was identical in all cases and only the contact time with 5-HT was varied within this set period. Inhibitors were allowed 3 min contact before addition of the agonist, unless otherwise specified.

Drugs used were adenosine 5′-diphosphate (Sigma), 5-hydroxytryptamine creatinine sulphate (May & Baker), adrenaline acid tartrate (BDH), imipramine hydrochloride (ICI), phentolamine hydrochloride (Ciba-Geigy), (+)-propranolol hydrochloride (ICI) and methysergide bimaleate, gift from Sandoz, Ltd., Basle. All drugs were freshly dissolved in 0.9% w/v NaCl solution (saline) and added to PRP in volumes not exceeding 20 µl per 1 ml PRP.

Results

Enhancement of adenosine diphosphate-induced aggregation by 5-hydroxytryptamine

Both human and rabbit platelets aggregate in response to ADP in a concentration-dependent manner. Human platelets are aggregated by 5-HT but the aggregates are smaller and disaggregate more rapidly than aggregates produced by equal concentrations of ADP. When 5-HT and ADP are added simultaneously to stirred PRP, the resulting aggregation is faster than that caused by either of the aggregating agents alone. 5-HT alone does not aggregate platelets in rabbit citrated PRP, but as seen in human PRP, it increases the aggregating effect of ADP (Sinakos & Caen, 1967). Figure 1 shows the effect of four concentrations of 5-HT on the rate and extent of aggregation of rabbit platelets produced by different concentrations of ADP. The velocity as well as the extent of aggregation, i.e. reduction in number of single platelets (Born & Hume, 1967; Cronberg, 1970), which is measured by increase in light transmission are enhanced in the presence of 5-HT. The enhancement of aggregation is greatest with low concentrations of ADP, i.e. 0.2–1 µM when the rate and extent of aggregation are increased six to seven fold by the addition of 5-HT 1–5 µM. At higher ADP concentrations the enhancement by 5-HT is less. In rabbit PRP, ADP produces maximal velocity of aggregation at concentrations of 5–10 µM, and therefore no potentiating effect of 5-HT can be measured.

Enhancement of adenosine diphosphate-induced shape change by 5-hydroxytryptamine

The rapid morphological change of platelets, which precedes their aggregation, is induced by 5-HT or by ADP in both rabbit and human plasma. The rate as well as the extent of shape change, i.e. the number of platelets with changed shape, depends on the concentration of the aggregating agents and generally occurs with concentrations well below those needed to

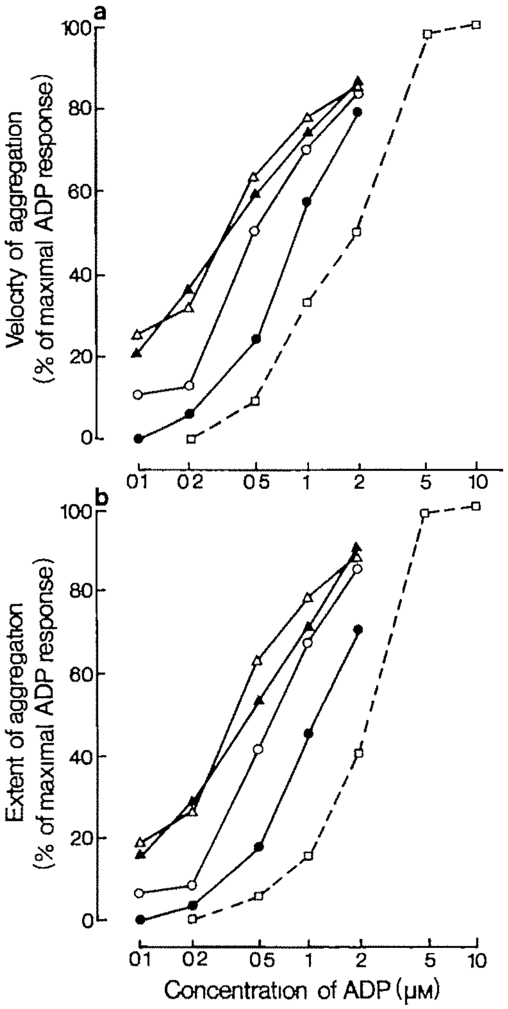


Figure 1 Potentiation by 5-hydroxytryptamine (5-HT) of platelet aggregation induced in rabbit platelet-rich plasma by adenosine diphosphate (ADP) Plots of (a) velocity of aggregation and (b) extent of aggregation 5-HT was injected simultaneously with ADP in volumes of 10 μl per 1 ml plasma. (□) ADP alone; (●) ADP with 5-HT 0.5 μM; (○) ADP with 5-HT 1 μM; (▲) ADP with 5-HT 5 μM and (△) ADP with 5-HT 20 μM. Stirred platelet-rich plasma was maintained at 37°C and aggregation was measured by recording changes in light transmission

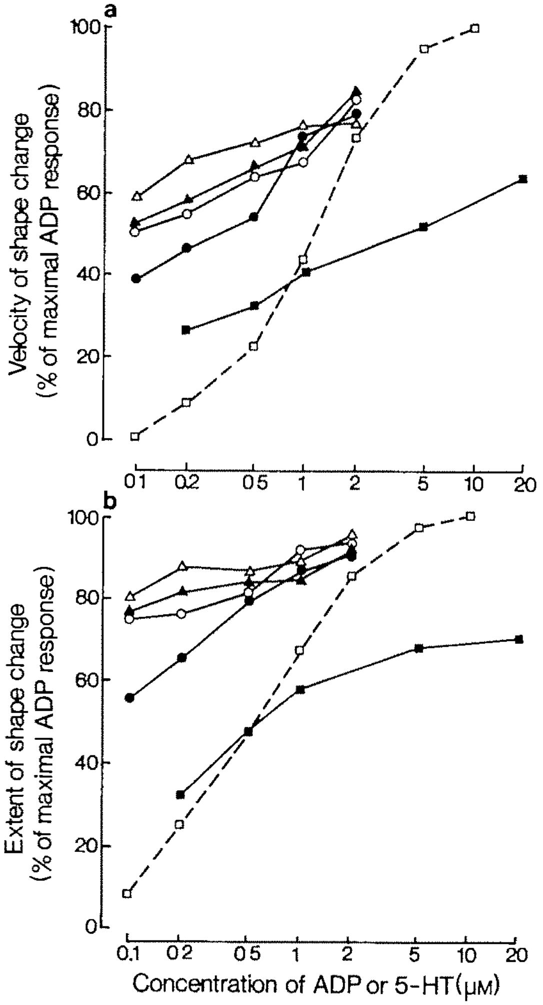


Figure 2 Potentiation of adenosine diphosphate (ADP)-induced shape change of rabbit platelets by 5-hydroxytryptamine (5-HT). Plots of (a) velocity of shape change and (b) extent of shape change. 5-HT was injected simultaneously with ADP. (□) ADP alone; (■) 5-HT alone, (●) ADP with 5-HT 0.5 μM; (○) ADP with 5-HT 1 μM, (▲) ADP with 5-HT 5 μM, (△) ADP with 5-HT 20 μM. Shape change was measured by recording changes in light scattering through stirred platelet-rich plasma at 37°C.

induce aggregation (Figure 2). The results were obtained in rabbit PRP by measuring 90° light scattering by platelets (Michal & Born, 1971). When 5-HT and ADP are injected together into PRP, the velocity and the extent of the morphological change is always greater than that produced by either agent alone. Concentrations of 5-HT which by themselves produce no detectable response, when added together with ADP bring about a rapid shape change of platelets. In rabbit PRP, the effect produced by low concentrations of ADP is markedly increased by very low concentrations of 5-HT (0.1–0.5 μM). Results of typical experiments are shown in Figure 3. As in the case of aggregation, 5-HT causes a greater enhancement of the effect of low concentrations of ADP (0.1–0.5 μM) than of concentrations of ADP which by themselves induce near maximal shape change (1–2 μM).

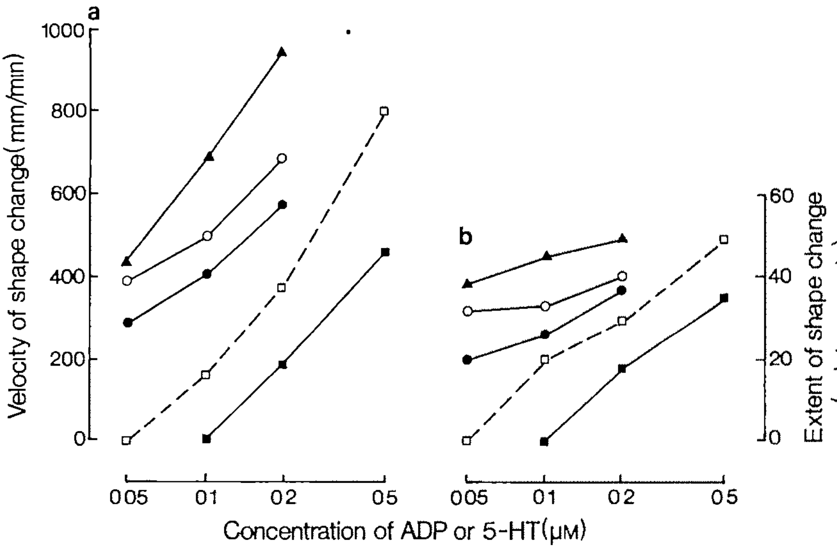


Figure 3 Potentiation by low concentrations of 5-hydroxytryptamine (5-HT) of platelet shape change caused by low concentrations of adenosine diphosphate (ADP) in rabbit platelet-rich plasma. Effect on (a) velocity of platelet shape change and (b) extent of shape change (□) ADP alone, (■) 5-HT alone; (●) ADP with 5-HT 0.1 µM; (O) ADP with 5-HT 0.2 µM, (▲) ADP with 5-HT 0.5 µM 5-HT was injected simultaneously with ADP.

Effect of inhibitors on aggregation and shape change

Incubation of PRP for 3 min at 37°C with methysergide (0.25 µM) before the addition of the aggregating agents (ADP and 5-HT) abolishes the enhancing effect of 5-HT on ADP (Figure 4). Methysergide at this concentration has no inhibitory effect on aggregation produced by ADP. Imipramine (0.5 µM) reduces the effect of 5-HT on ADP aggregation only slightly. A mixture of imipramine (0.5 µM) and methysergide (0.25 µM) has the same inhibitory effect as methysergide alone.

Similarly the increase by 5-HT of the velocity of shape change produced by ADP in human platelets is abolished by methysergide (0.25 µM); imipramine (0.5 µM) reduces the effect only slightly (Figure 5).

Table 1 The effect of pre-incubation of platelets with 5-hydroxytryptamine (5-HT) on their response to adenosine diphosphate (ADP) and potentiation by 5-HT of aggregation by ADP

Pre-incubation (min) with 5-HT (5 µM)	*ADP alone (0.5 µM) or ADP (0.5 µM) + 5-HT (10 µM)*	*Aggregation (% ADP control)** *Velocity*	*Extent*
	Rabbit PRP		
No pre-incubation	ADP + 5-HT	145	152
3	ADP	120	122
	ADP + 5-HT	121	123
10	ADP	74	81
	ADP + 5-HT	86	92
	Human PRP		
No pre-incubation	ADP + 5-HT	191	272
3	ADP	74	59
	ADP + 5-HT	58	50
10	ADP	30	34
	ADP + 5-HT	32	32

* Aggregation by ADP alone at time zero is expressed as 100%.

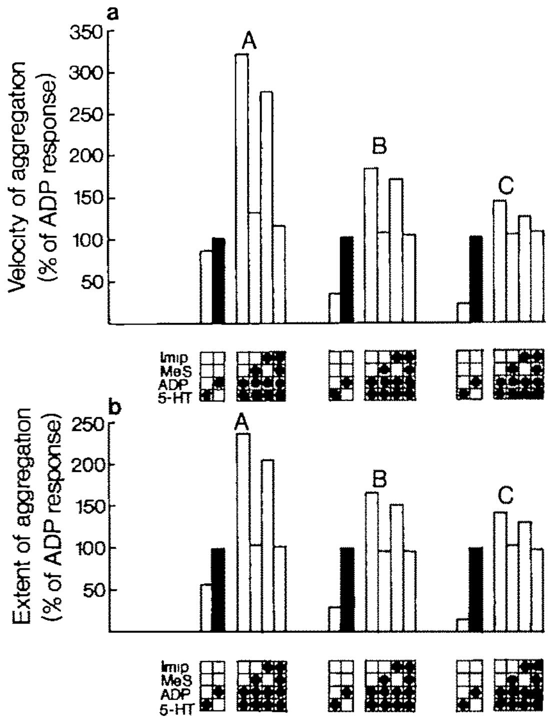


Figure 4 Effect of 0.5 µM imipramine (Imip) and 0.25 µM methysergide (MeS) on the potentiation by 5-hydroxytryptamine (5-HT, 0.5 µM) of (a) velocity and (b) extent of aggregation induced in human platelet-rich plasma by adenosine diphosphate (ADP). Concentrations of ADP: A=0.5 µM; B=1 µM, C=2 µM 5-HT and ADP were injected simultaneously. Control responses to ADP alone (solid columns) were given a value of 100% The inhibitiors were incubated in the platelet suspension for 3 min before addition of the ADP/5-HT mixture

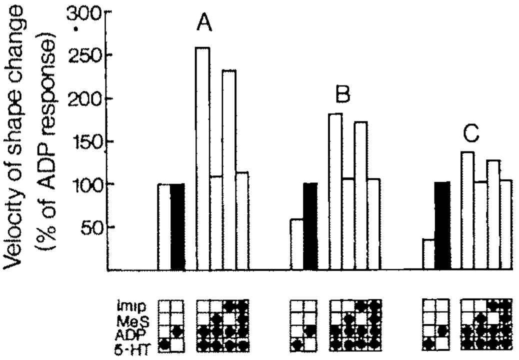


Figure 5 Effect of 0.5 µM Imipramine (Imip) and 0.25 µM methysergide (MeS) on the potentiation by 5-hydroxytryptamine (5-HT, 0.5 µM) of the velocity of shape change induced in human platelet-rich plasma by adenosine diphosphate (ADP) (A=0.5 µM, B=1 µM and C=2 µM ADP) 5-HT and ADP were injected simultaneously Control responses to ADP alone were given a value of 100% (solid columns). The inhibitors were incubated in the platelet-rich plasma for 3 min before the addition of the ADP/5-HT mixture

Inhibition by 5-hydroxytryptamine itself

Enhancement of ADP-induced platelet aggregation by 5-HT is greatest when the two agents are injected into PRP simultaneously. When 5-HT is injected before ADP, the enhancing effect is smaller. By increasing the time of pre-incubation with 5-HT, not only the enhancing effect of 5-HT disappears but the effect of ADP is progressively reduced. Table 1 shows the effect of pre-incubating rabbit or human platelets with 5 µM 5-HT for 3 or 10 min before adding either ADP alone or a mixture of ADP and 5-HT. In rabbit PRP the velocity and extent of ADP aggregation after 3 min pre-incubation with 5-HT are still greater than aggregation caused by ADP alone; however, after 10 min, the velocity and extent are reduced. In human PRP pre-incubated with 5-HT for 3 min, aggregation by ADP or ADP plus 5-HT is inhibited, and after 10 min pre-incubation, velocity and extent are further reduced to approximately 30% of the control.

Platelet refractoriness to adenosine diphosphate and 5-hydroxytryptamine

Platelets are made refractory to ADP by repeated aggregation with ADP (Table 2). After the fourth injection of ADP (9 min), the extent of aggregation in human PRP is only 13–15% of the aggregation obtained with the first addition of ADP; injection of 5-HT into this PRP at time 12 min produces extensive aggregation which is twice as great as the 5-HT control. Methysergide (0.5 µM) inhibited the action of 5-HT only.

Platelets become rapidly refractory to repeated injections of 5-HT; a second injection of 5-HT given 3 min after the first fails to aggregate platelets (Table 2). However, sensitivity of these platelets to ADP remains unchanged, including the gradual development of refractoriness to repeated additions of this agent.

Rabbit platelets are also made refractory to ADP by repeated injections of this agent (Table 2). After three injections of ADP (1 µM) made at 3 min intervals, the platelets are no longer aggregated by ADP. However, when 5-HT is injected into the same PRP, aggregation is produced (29% of control) despite the inability of 5-HT to aggregate rabbit platelets that have not been exposed to ADP; subsequent additions of 5-HT produce no aggregation. The aggregating

Table 2 Platelet refractoriness to multiple injections of adenosine diphosphate (ADP, 1 μM) or 5-hydroxytryptamine (5-HT, 10 μM) in human or rabbit platelet-rich plasma (PRP)

		*Extent of aggregation (% ADP control)** *Time of injection (min)*					
Inhibitor	*Substance injected*	0	3	6	9	12	24
	Human PRP						
	ADP	100	47	19	13	5	6
	5-HT	67	0	0	0	0	
	ADP	100	68	29	15		3
	5-HT					200	
Methysergide (0.5 μM)	ADP	100	58	24	15		4
	5-HT					0	
	ADP			95	47	19	6
	5-HT	68	0				
	Rabbit PRP						
	ADP	100	7	0			0
	5-HT				29	0	
Methysergide (0.5 μM)	ADP	100	10	0			0
	5-HT				0	0	
Imipramine (0.5 μM)	ADP	100	9	0			0
	5-HT				28	0	
	ADP			96	26	0	
	5-HT	0	0				

* Aggregation by ADP alone at time zero is expressed as 100%. Inhibitors were pre-incubated in PRP for 3 minutes.

effect of 5-HT produced in platelets exposed to ADP is inhibited by methysergide (0.5 μM) but not by imipramine (0.5 μM). In rabbit platelets which do not aggregate after two injections of 5-HT given at 3 min intervals, addition of ADP results in normal aggregation and subsequent development of the refractory effect (Table 2).

Shape change, like aggregation, diminishes after repeated injections of ADP and it is produced only by the first injection of 5-HT. Platelets made refractory to 5-HT change shape with ADP, and platelets made partially refractory to the shape changing action of ADP respond to addition of 5-HT.

Secondary induction of aggregation or shape change

When rabbit or human platelets are aggregated with ADP, the change in light transmission can be further increased by a second addition of ADP providing that the initial concentration of ADP causes a submaximal aggregating response and that the second addition is made at the peak of the initial aggregation

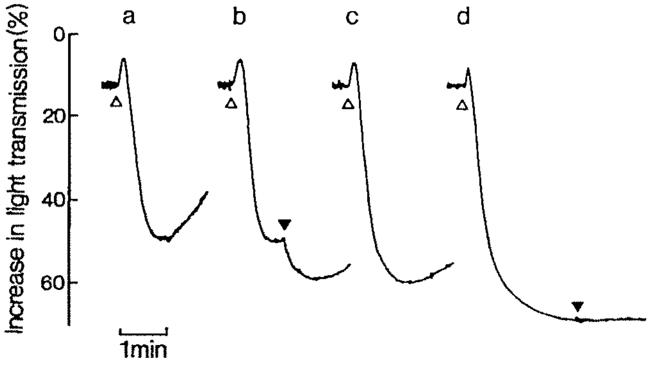


Figure 6 Change in light transmission in rabbit platelet-rich plasma caused by platelet aggregation induced by adenosine disphosphate (ADP, △): (a) 1 μM; (b) 1 μM; (c) 2 μM, (d) 10 μM; followed by 1 μM ADP (▲) in (b) and (d).

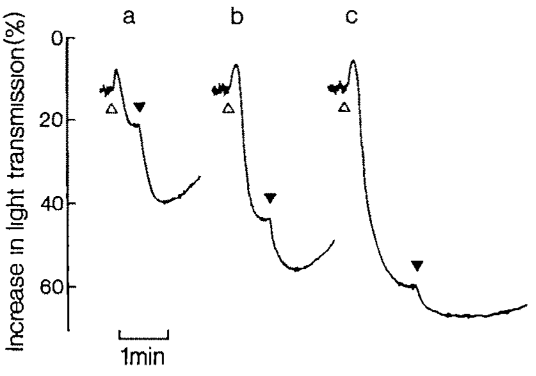


Figure 7 Secondary induction of aggregation by 10 μM 5-hydroxytryptamine (▲) of platelets in rabbit platelet-rich plasma aggregated initially by adenosine diphosphate (△): (a) 0.5 μM, (b) 1 μM and (c) 2 μM.

(Figure 6). The final extent of aggregation produced in this way is similar to aggregation caused by the combined amount of ADP added in one single injection. 5-HT injected as the second addition after ADP produces a similar increase in the aggregating effect (Figure 7). This additional effect is shown not only in human but also in rabbit PRP where 5-HT by itself does not aggregate platelets. Similarly, adrenaline which does not aggregate rabbit platelets nevertheless causes additional aggregation when added after ADP (Figure 8a).

The specificity of the additional aggregation was tested by incubating the PRP samples with antagonists of 5-HT and adrenaline for 3 min before aggregation. Pre-incubation with phentolamine (5 μM) removes the additional aggregation superimposed on ADP by adrenaline 10 μM (Figure 8f); propranolol (5 μM), imipramine (0.5 μM) and methysergide (0.25 μM) are inactive (Figure 8c, d and g). Incubation with methysergide abolishes the additional aggregating effect caused by 5-HT after the induction of aggregation by ADP (Figure 9d); imipramine, phentolamine and propranolol are inactive (Figure 9c, f and g).

Platelets aggregated by ADP do not show additional aggregation with 5-HT if they are pre-incubated for 3 min with 5-HT itself although the velocity and extent of initial aggregation by ADP are greater than without 5-HT pre-incubation (Figure 9e). This lack of additional aggregation in PRP samples pre-incubated with 5-HT resembles the self-inhibition by 5-HT of its potentiating effect on ADP already described (see Table 1). The self-inhibition is specific; pre-incubation with 5-HT does not inhibit the additional aggregation produced by either adrenaline or ADP (Figure 8e, Figure 10e).

Pre-incubation of PRP with adrenaline for 3 min before the addition of ADP increases the extent of platelet aggregation by ADP; such pre-incubation inhibits the effect of adrenaline added after ADP (Figure 8b) but not that of 5-HT (Figure 9b).

The shape change of platelets produced by ADP is increased when 5-HT and ADP are injected together or by injecting 5-HT at the peak of the ADP shape change in the presence of ethylene glycol bis-(2-aminoethyl) tetra-acetic acid (4 mM) to prevent aggregation. Methysergide, but not imipramine, removes the effect of 5-HT leaving the ADP shape change undiminished. Pre-incubation of platelets with 5-HT for 3 min prevents the effect of the second addition of 5-HT but the shape change produced by ADP is not inhibited. In this respect, aggregation and shape change resemble each other.

Adrenaline failed to induce an additional effect when injected at the peak of ADP-induced shape

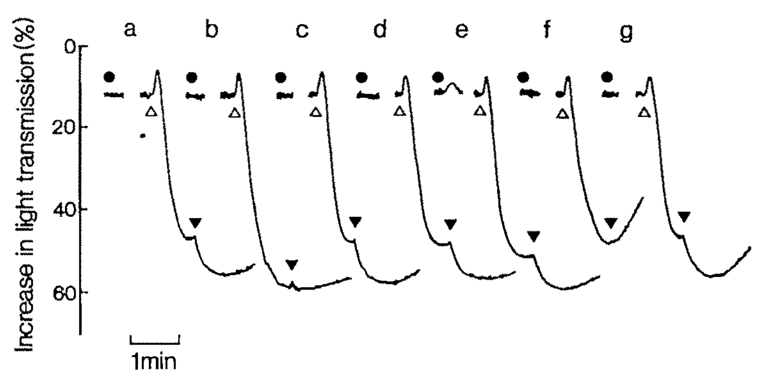


Figure 8 Effect of inhibitors on the secondary induction of aggregation of platelets in rabbit platelet-rich plasma caused by 10 μM adrenaline (▲) added after 1 μM adenosine diphosphate (△). Platelets were pre-incubated for 3 min (●) with: (a) saline control; (b) adrenaline 10 μM; (c) imipramine 0.5 μM; (d) methysergide 0.25 μM; (e) 5-hydroxytryptamine 10 μM; (f) phentolamine 5 μM; and (g) propranolol 5 μM.

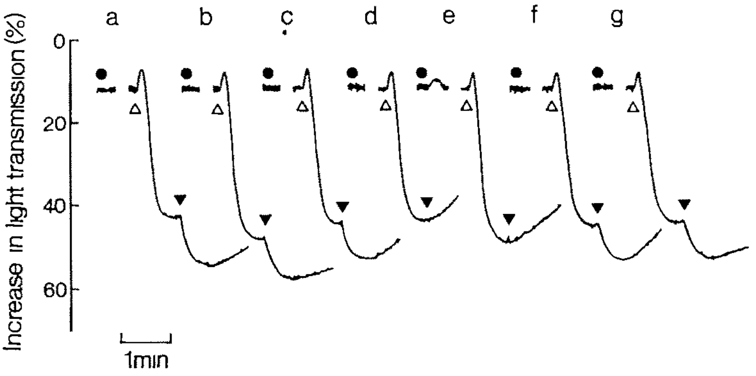


Figure 9 Effect of inhibitors on the secondary induction of aggregation by 5-hydroxytryptamine (5-HT) 5 µM (▲) of rabbit platelets aggregated by adenosine diphosphate 1 µM (△). Plasma was pre-incubated (●) for 3 min with: (a) saline control; (b) adrenaline 1 µM; (c) imipramine 0 5 µM; (d) methysergide 0.25 µM; (e) 5-HT 5 µM; (f) phentolamine 5 µM and (g) propranolol 5 µM

change although adrenaline injected together with ADP did increase the shape change slightly. This lack of response to adrenaline is probably due to its inability to produce a rapid shape change; the shape change produced by adrenaline had a much slower time course than that produced by ADP or 5-HT.

Discussion

In some species including man, cat, dog and sheep, 5-HT causes platelets to aggregate more rapidly and completely than in other species, such as the guinea-pig and rat (Mills, 1970). The mechanism by which 5-HT causes aggregation is not yet clearly understood except that it is probably mediated via receptors similar to the D-receptors for 5-HT on smooth muscle (Michal, 1969). At one time the receptors responsible for aggregation seemed identical to those mediating 5-HT transport (Baumgartner & Born, 1969). More recent evidence indicates that the uptake and aggregating receptors are different (Born *et al.*, 1972).

The initial and immediate response of platelets to 5-HT is a rapid change in their shape, even when aggregation is not produced. This shape change can be caused by analogues of 5-HT which are not taken up by the platelets (Born *et al.*, 1972). Antagonists of 5-HT which inhibit the morphological change also inhibit aggregation by 5-HT, so that these effects are presumably mediated through the same receptor.

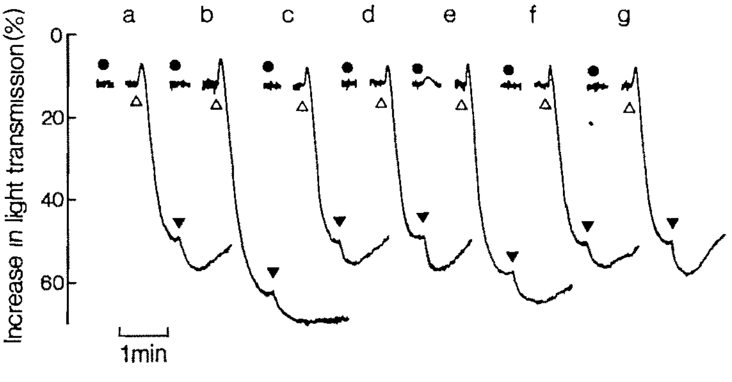


Figure 10 Effect of inhibitors on aggregation of rabbit platelets by adenosine diphosphate (ADP) 1 µM (△) followed by a second addition of ADP 1 µM (▲). Platelet-rich plasma was pre-incubated for 3 min (●) with: (a) saline control; (b) adrenaline 1 µM; (c) imipramine 0.5 µM; (d) methysergide 0.25 µM, (e) 5-HT 10 µM; (f) phentolamine 5 µM and (g) propranolol 5 µM.

Potentiation of one naturally occurring aggregating agent by another can be clearly demonstrated *in vitro*. Such interaction could also occur under *in vivo* conditions. We have demonstrated that the aggregating effect of ADP can be greatly potentiated *in vitro* by small concentrations of 5-HT. This may be of physiological importance because ADP is thought to be one of the substances causing platelet adhesion and aggregation *in vivo*. Concentrations of 5-HT and ADP which have no effect by themselves, are able to aggregate platelets when present in combination. In still smaller concentrations, these agents injected together can induce platelet shape change alone. The significance of the platelet shape change is not entirely clear, although platelets which do not change shape are defective in haemostatic function (Caen & Michel, 1972).

5-HT stored in platelets is liberated *in vitro* from the storage sites by collagen or thrombin and also by high concentrations of ADP. This release, which is an energy consuming process, is a characteristic platelet reaction and could be important in platelet behaviour *in vivo*. It has been demonstrated, for example, that 5-HT released from platelets during aggregation can accelerate and consolidate a platelet plug and formation of thrombus (Michal & Penglis, 1969).

The enhancing effect of 5-HT on shape change and aggregation induced by ADP seems to be caused by the action of 5-HT at plasma membrane receptor. Methysergide, but not imipramine, inhibits the potentiating action of 5-HT just as it blocks the direct action of the amine itself. These results indicate that 5-HT produces these effects via receptors different from those responsible for 5-HT uptake.

Platelets made refractory to ADP can still aggregate and change shape when exposed to 5-HT. In a similar manner platelets refractory to 5-HT aggregate and change shape in the presence of ADP. The effect of 5-HT is prevented by methysergide but not by imipramine. The additional aggregating and shape changing effect cannot be explained by simple synergism between the effects of the residual amounts of one agonist and the addition of another because under these conditions platelets are not sensitive to additions of high concentrations of the same agonist. These observations suggest that the effects of ADP and 5-HT are mediated by different mechanisms and that the refractory state induced through paralysis of one of these mechanisms need not prevent the effect of a different agonist. These results also support the suggestion by Evans & Gordon (1974) that refractoriness, at least as far as aggregation is concerned, does not represent a non-specific depression of platelet reactivity.

5-HT increased the extent of aggregation which had already been initiated by ADP provided that the initial effect of ADP was submaximal. Here too, methysergide specifically prevented the action of 5-HT and not that of ADP or adrenaline. Similarly, adrenaline potentiated the aggregation caused by ADP and this potentiating effect was antagonized by phentolamine; propranolol, methysergide and imipramine did not inhibit it. The additional aggregating effect caused by either adrenaline or 5-HT is therefore a specific and characteristic action of each of these substances.

These results show that platelet aggregation may be produced through stimulation of different receptors (by ADP, 5-HT or adrenaline) and that the effects can combine to give the overall response observed.

Under certain conditions, prior contact of platelets with 5-HT can render them resistant to the effect of further additions of 5-HT or other aggregating agents (Baumgartner & Born, 1969). Our results confirm these earlier observations. This inhibitory effect of 5-HT follows a time course similar to that of the active uptake process although uptake inhibitors, such as imipramine, do not interfere with the interaction of ADP and 5-HT effects on platelet aggregation and shape change. The situation is a complex one and it is not yet clear why the observed effect of 5-HT on platelets can be sometimes that of an agonist and at other times that of an antagonist or a 'stabilizing agent'. Nevertheless, this increase or depression of platelet reactivity appears to be a highly specific effect and is probably mediated at specific receptors involved with platelet activation.

F.M. is a Senior Research Fellow of the British Heart Foundation and M.M. is in receipt of a fellowship from the Wellcome Trust.

References

BAUMGARTNER, H.R. & BORN, G.V.R. (1968). Effect of 5-HT on platelet aggregation. *Nature, Lond.*, **218**, 137–141.

BAUMGARTNER, H.R. & BORN, G.V.R. (1969). The relation between the 5-HT content and aggregation of rabbit platelets. *J. Physiol., Lond.*, **201**, 397–408.

BORN, G.V.R. & GILLSON, R.E. (1959). Studies on the uptake of 5-HT by blood platelets. *J. Physiol., Lond.*, **146**, 472–491.

BORN, G.V.R. & HUME, M. (1967). Effect of the numbers and sizes of platelet aggregates on the optical density of plasma. *Nature, Lond.*, **215**, 1027–1029.

BORN, G.V.R., INGRAM, G.R.C. & STACEY, R.S. (1958). The relationship between 5-HT and ATP in blood platelets. *Br. J. Pharmac. Chemother.*, **13**, 62–64.

BORN, G.V.R., JUENGJAROEN, K. & MICHAL, F. (1972). Relative activities on and uptake by human blood platelets of 5-hydroxytryptamine and several analogues. *Br. J. Pharmac.*, **44**, 117–139.

CAEN, J.P & MICHEL, H. (1972). Platelet shape change and aggregation. *Nature, Lond.,* **240**, 148–149.

CRONBERG, S. (1970). Evaluation of platelet aggregation. *Coagulation,* **3**, 139–151.

DA PRADA, M & PLETSCHER, A. (1968). Isolated 5-HT organelles of rabbit blood platelets. Physiological properties and drug-induced changes. *Br. J. Pharmac.,* **34**, 591–597.

EVANS, R.J. & GORDON, J.L. (1974). Refractoriness in blood platelets: effect of prior exposure to aggregating agents on subsequent aggregation responses. *Br. J. Pharmac.,* **51**, 123P.

GRETTE, K. (1962). Studies on the mechanism of thrombin-catalysed haemostatic reaction in blood platelets. *Acta physiol. scand.,* **56**, suppl. 195.

HARDISTY, R.M. & STACEY, R.S. (1955). 5-Hydroxytryptamine in normal human platelets. *J. Physiol., Lond.,* **130**, 711–720.

MARMARAS, V.J. & MIMIKOS, N. (1971). Enzymic formation of serotonin in mammalian blood platelets and red cells. *Experientia,* **27**, 196–197.

MICHAL, F. (1969). D-Receptor for serotonin on blood platelets. *Nature, Lond.,* **221**, 1253–1254.

MICHAL, F. & BORN, G.V.R. (1971). Effect of the rapid shape change of platelets on the transmission and scattering of light through plasma. *Nature, New Biol.,* **231**, 220–222.

MICHAL, F. & PENGLIS, F. (1969). Inhibition of serotonin-induced platelet aggregation in relation to thrombus production. *J. Pharmac. exp. Ther.,* **166**, 266–284.

MILLS, D C.B. (1970). Platelet aggregation and platelet nucleotide concentration in different species. *Symp. Zool. Soc. Lond.,* **27**, 99–107.

MILLS, D.C.B. & ROBERTS, J.C.K. (1967). Membrane active drugs and the aggregation of human blood platelets. *Nature, Lond.,* **213**, 35–38.

MUSTARD, J.F. & PACKHAM, M.A (1970). Factors influencing platelet function, adhesion, release and aggregation. *Pharmac. Rev.,* **22**, 97–187.

PAASONEN, M.K. (1965). Release of 5-hydroxytryptamine from blood platelets. *J. Pharm. Pharmac.,* **17**, 681–697.

PACKHAM, M.A., GUCCIONE, M.A., CHANG, P. & MUSTARD, J.F. (1973). Platelet aggregation and release: effects of low concentrations of thrombin and collagen. *Amer. J. Physiol.,* **225**, 38–47.

PLETSCHER, A., DA PRADA, M. & TRANZER, J.P. (1969). Transfer and storage of biogenic monoamines in subcellular organelles of blood platelets. *Progr. Brain Res.,* **31**, 47–52.

RAND, M. & REID, G (1951). Source of serotonin in serum. *Nature, Lond.,* **168**, 385.

SINAKOS, Z. & CAEN, J.P. (1967). Platelet aggregation in mammalians (Human, Rat, Rabbit, Guinea-Pig, Horse, Dog). A comparative study. *Thromb. Diath. haemorrh.,* **17**, 99–111.

SNEDDON, J M. (1973). Blood platelets as a model for monoamine-containing neurones. In *Progress in Neurobiology,* vol. 1 part 2. pp. 151–198. ed. Kerkut, G.A. & Phillis, J.W. Oxford & New York: Pergamon Press.

(Received October 14, 1975.)

Br. J. Pharmac. (1975), **55**, 219–227

CARDIOVASCULAR EFFECTS OF DIETHYLCARBAMAZINE CITRATE

A.K. ABAITEY & J.R. PARRATT

The Department of Physiology & Pharmacology, Royal College, University of Strathclyde,
Glasgow G1 1XW

1 The cardiovascular effects of the anthelmintic drug diethylcarbamazine citrate (DECC) were examined in cats anaesthetized with pentobarbitone. There were two quite distinct haemodynamic responses, an initial transient hypotension (occurring within 10 s of an intravenous injection) and a pronounced secondary hypertension which reached a peak 30–60 s after the injection.

2 Within 10 s of an intravenous injection of DECC (2.5 to 10 mg/kg) there was hypotension, bradycardia and there were reductions in left ventricular and carotid artery d*P*/d*t* *max*. These effects were most pronounced following injections into the pulmonary artery; they were not observed after bilateral vagotomy or after injections into the lumen of the left ventricle. It is suggested that DECC, like nicotine, stimulates vagal receptors in the pulmonary vascular bed.

3 The secondary phase was characterized by marked systemic and pulmonary hypertension, by contractions of the nictitating membrane and by increases in left ventricular d*P*/d*t* (at fixed isovolumic pressures), in cardiac output and in myocardial blood flow. All these effects were prevented, or markedly reduced, following the administration of hexamethonium or bethanidine and the pressor response was prevented by phentolamine. It is concluded that, in doses similar to those used in therapeutics, DECC stimulates sympathetic ganglia and releases noradrenaline. The relevance of this finding to the reported side effects of the drug are discussed.

4 DECC (5 or 10 mg/kg) significantly inhibited prostaglandin $F_{2\alpha}$-induced increases in peak inspiratory intra-tracheal pressure without modifying its pulmonary hypertensive effect. The possible relevance of this finding to the use of DECC in asthma is discussed.

Introduction

The anthelmintic diethylcarbamazine citrate (DECC) is the drug of choice in the treatment of *Wuchereria bancrofti*, *Brugia malayi* and *Loa loa*. It has been administered to millions of people and is generally regarded as a safe drug at therapeutic levels. Most of the side effects associated with its use have been attributed to the release of foreign protein from dying microfilariae and adult worms (Goldsmith, 1972). More recently, DECC has been used, with varying results, in the treatment of bronchial asthma (Mallen, 1965; Srinivas & Antani, 1971; Koivikko, 1973; Sly & Matzen, 1974).

Although only a few adverse reactions (chiefly nausea, vomiting and headache) have been directly attributed to the drug, Oomen (1969) has reported, from one hospital in Ethiopia, coma and death in seven out of 56 severely debilitated patients being treated for onchocerciasis. This reaction was apparently not allergic in nature. More recently, Fuglsang & Anderson (1974) have also emphasized that great care be taken when the drug is used to treat patients heavily infected with *Onchocerca volvulus*. In their study, from Cameroun, the more serious adverse reactions included a weak pulse, respiratory distress and coma; these reactions were partly attributed to the presence of microfilariae in the circulation.

Apart from the initial study of Harned, Cunningham, Halliday, Vessey, Yuda, Clark & Subba Row (1948) and a brief report by Forbes (1972), there have been no studies of the effects of DECC on the circulation. There have been no published accounts of effects of the drug on cardiac contractility either *in vivo* or *in vitro*. However, it is of some interest in this connection that Harned *et al.* (1948) reported sinus arrest, tachycardia and premature ventricular contractions following intravenous administration of the drug into conscious dogs. In the present paper we describe in detail the effects of DECC on systemic and pulmonary pressures, on myocardial contractility and blood flow, and on the electrocardiogram of anaesthetized cats.

Methods

Anaesthetized cats

Twenty-one cats, weighing between 1.5 and 3.5 kg, were anaesthetized with sodium pentobarbitone

(30 mg/kg, i.p.) and respired with room air by means of a Palmer positive-pressure ventilation pump (rate 20/min; stroke volume 40–60 ml). The volume of the pump was adjusted to give an arterial $P\text{O}_2$ between 80 and 100 mmHg (measured with a calibrated Radiometer electrode, for details see Parratt, 1973). A certain degree of hyperventilation was necessary (arterial $P\text{CO}_2$ 26 ± 2 mmHg); in spontaneously breathing cats anaesthetized with pentobarbitone it is 33 ± 1 mmHg (Parratt, 1973). Changes in respiration were assessed in spontaneously breathing cats with a copper-constantan thermocouple (similar to that used to record intramyocardial temperature; see below) placed within the tracheal cannula. The rate and depth of respiration were assessed from the changes in the respired air temperature and were recorded on a Kipp 2 Zonen BD5 recorder (full scale 200 mm = 1 mV = 25°C).

Systemic arterial blood pressure was recorded with a capacitance transducer (Elema-Schönander EMT 35) from a catheter, inserted by way of the carotid or femoral artery, so that the tip lay in the descending aorta or aortic arch. Systolic ejection time was measured (using a paper speed of 250 mm/s) from the beginning of the upstroke of the central aortic pressure pulse to the trough of the incisural notch and carotid artery $\mathrm{d}P/\mathrm{d}t$ was continuously determined with an Elema-Schönander differentiating circuit. Mean arterial pressure was obtained by electronic integration.

After catheterizing a femoral vein, heparin (200 units/kg) was administered intravenously and a left thoracotomy performed. A wide-bore steel needle was inserted into the lumen of the left ventricle by direct puncture of the wall and pressure recorded with a capacitance transducer (EMT 34) both at high gain (10 mmHg = 25 mm; 1 mmHg = 1.333 mbar) for the accurate assessment of left ventricular end-diastolic pressure (LVEDP) and, at a lower gain (100 mmHg = 25 mm) for recording the full left ventricular pressure pulse. As an index of myocardial contractility the rate of rise of left ventricular pressure with time ($\mathrm{d}P/\mathrm{d}t$) was determined with an Elema-Schönander differentiating circuit and, to correct for changes in afterload (Mason, 1969), plots of $\mathrm{d}P/\mathrm{d}t$ at different isovolumic pressures were obtained using a paper speed of 1000 mm/second. Either right atrial pressure (using a third capacitance transducer (EMT 33) from a catheter inserted by way of the right external jugular vein) or pulmonary artery pressure (from an indwelling needle in the main pulmonary artery) was also recorded, together with the ECG (usually limb leads I or II) on an eight-channel ink-jet writing recorder (Elema-Schönander mingograph 81).

In some of the experiments intratracheal pressure was measured from a side arm of the tracheal cannula with a Statham P23D6 pressure transducer. Cardiac output was measured by thermal dilution with room temperature 0.9% w/v NaCl solution (saline) (for details see Parratt, 1973) and myocardial blood flow was assessed by means of heated thermocouples implanted in the wall of the left ventricle. Because of the transient nature of the responses a constant heating current (0.15 A^2) was used as described by Parratt & Wadsworth (1972).

In three experiments the contractions of the nictitating membrane in response to pre-ganglionic sympathetic nerve stimulation were recorded with a Grass (model FT03) strain-gauge and a George Washington pen recorder (400 MD/2). Trains of rectangular pulses (0.5 ms duration) were delivered from a Grass S88 stimulator (frequency 10 Hz for 10 s at 100 ms intervals).

Diethylcarbamazine citrate (DECC; from Dr. A.F. Green, The Wellcome Research Laboratories) was dissolved in saline and injected, in doses of 2.5 (9 injections), 5.0 (17 injections) and 10 (36 injections) mg/kg (as salt) either intravenously or directly into the pulmonary artery or left ventricle.

Isolated coronary artery strips

Coronary arteries (o.d. 1.5–3.0 mm) were removed from pig hearts and cut into spiral strips approximately 3 cm long. They were suspended in Krebs solution, gassed with carbogen, at 37°C; the resting tension was 1 gram. Contractions were induced with potassium chloride (10–20 mM) and recorded isometrically.

Results

Haemodynamic effects in anaesthetized cats

There were two quite distinct haemodynamic responses to the injection of DECC: an initial transient hypotension (occurring within 10 s of the injection) and a pronounced secondary hypertension reaching a peak 30–60 s after the injection. These two phases (which are illustrated in Figure 1) will be described separately.

Initial hypotensive effect of diethylcarbamazine citrate Within 4–5 s of a rapid intravenous injection of DECC (2.5–10.0 mg/kg) there was bradycardia and slight reductions in systemic and pulmonary arterial pressures and also in left ventricular and carotid artery $\mathrm{d}P/\mathrm{d}t$ *max.*; there was usually no reduction in pulse pressure. This initial effect of DECC is illustrated in Figure 1 and the results of all the experiments are summarized in Table 1. This initial effect of DECC was transient (lasting 3–6 s) and was observed more frequently with the higher doses of DECC. For example, it occurred in only 2 out of 9 experiments with a dose of 2.5 mg/kg, in 6 out of 17 experiments with a dose of 5.0 mg/kg and in 23 out of 36 experiments with 10 mg/kg. In

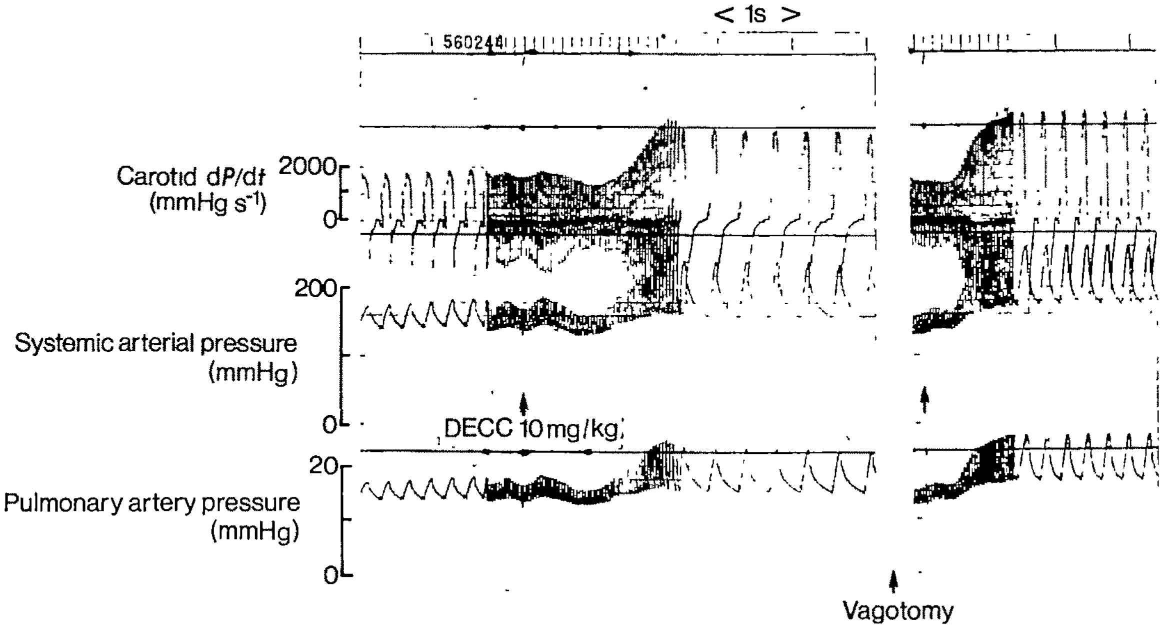


Figure 1 The effect, in an anaesthetized cat, of an intravenous injection of diethylcarbamazine citrate (DECC 10 mg/kg) on, from above, carotid artery dP/dt (mmHg s^{-1}) and on systemic and pulmonary artery pressures (mmHg) Within 10 s of the injection there was a transient hypotension and a reduction in dP/dt *max*.; this was followed by marked increases in pressure and dP/dt *max* and by cardiac slowing Both the initial hypotension and the secondary bradycardia were prevented by bilateral vagotomy (right hand panel).

spontaneously breathing cats this initial hypotension and bradycardia corresponded to a period of apnoea, lasting 7–10 seconds.

The effect of bilateral vagotomy Bilateral vagotomy itself had no significant haemodynamic effects in these pentobarbitone-anaesthetized cats (e.g. mean systemic blood pressure 96 ± 7 mmHg before and 91 ± 7 mmHg after vagotomy; heart rate 192 ± 7 beats/min before and 205 ± 10 beats/min after; carotid artery dP/dt *max*. 1690 ± 90 mmHg s^{-1} before and 1820 ± 110 mmHg s^{-1} after vagotomy; 10 experiments). In most of the experiments bilateral vagotomy greatly reduced the initial hypotensive effect of DECC (Figure 1). Thus in cats in which the effects of DECC (10 mg/kg) were examined both before and after vagotomy, the drug reduced mean blood pressure by 18 ± 4 mmHg (from 110 ± 12 mmHg) before vagotomy and by only 4–8 mmHg afterwards. Before vagotomy the heart rate change was −33 ± 12 beats/min (from a control of 196 ± 16 beats/min) but there was no reduction in heart rate following the administration of DECC in any experiment after vagotomy. The reduction in carotid artery dP/dt *max*.

Table 1 The initial (0–10 s) haemodynamic responses to the intravenous injection of diethylcarbamazine citrate (DECC, 2.5–10 mg/kg) in anaesthetized cats.

	Control	*Change from control induced by DECC*		
		2.5	*5.0*	*10.0 mg/kg*
Systolic blood pressure (mmHg)	124 ± 4	−7 (2)	−9 ± 1 (6)	−18 ± 2 (23)
Diastolic blood pressure (mmHg)	91 ± 4	−8 (2)	−9 ± 2 (7)	−19 ± 3 (23)
Carotid artery dP/dt *max* (mmHg s^{-1})	2200 ± 120	0 (8)	−430 ± 75 (6)	−560 ± 80 (23)
Left ventricular dP/dt *max*. (mmHg s^{-1})	3880 ± 800	0 (1)	−420 (2)	−890 ± 190 (5)
Heart rate (beats/min)	203 ± 8	0 (9)	−13 ± 3 (7)	−28 ± 5 (24)

The values are the mean changes from control ± s.e. with the number of observations in parentheses.

was unchanged by vagotomy (– 450 ± 115 mmHg s^{-1} before and – 590 ± 120 mmHg s^{-1} afterwards).

In one cat, in which the initial hypotension and bradycardia were particularly pronounced, it was possible to determine the effect of hexamethonium (0.5 mg/kg, i.v.) on this initial response. In this cat, DECC (10 mg/kg) reduced diastolic pressure by 39 mmHg (from 85 mmHg) and heart rate by 90 beats/min (from 200 beats/minute). After hexamethonium there was no decrease in pressure when DECC was injected and the heart rate change was only 10 beats/minute.

The effect of intrapulmonary and interventricular injections The effect of varying the route of administration of DECC was determined in three cats because of the evidence from *in vitro* studies (Abaitey & Parratt, unpublished) that the drug has nicotine-like actions; nicotine often induces a fall in systemic blood pressure through stimulation of sensory nerve endings in the lungs (Armitage & Hall, 1969).

When injected into the pulmonary artery, DECC (10 mg/kg) caused a decrease in left ventricular pressure, in mean systemic arterial pressure (a reduction of 18 ± 8 mmHg from a pre-injection level of 71 ± 2 mmHg), in heart rate (– 28 ± 5 beats/min from 204 ± 10 beats/min) and in left ventricular d*P*/d*t* *max.* (–1175 mmHg s^{-1} from a pre-injection level of 2230 ± 125 mmHg s^{-1}). These changes were similar to, but occurred 1–2 s sooner than, those observed in the same animals following the intravenous administration of DECC (10 mg/kg). When DECC was injected directly into the lumen of the left ventricle there was only a slight (< 10 mmHg) reduction in systemic pressure and the change in left ventricular d*P*/d*t* *max.* was much less marked (– 630 mmHg s^{-1}).

The secondary hypertensive effect of diethylcarbamazine citrate Within 10 s of the intravenous injection of DECC there were marked increases in systemic and pulmonary arterial pressures, in left ventricular d*P*/d*t* *max.* and, usually, also in carotid artery d*P*/d*t* *max.* These changes were dose related (Table 2) and the peak effect was reached in 30–60 seconds. At the height of the blood pressure response there was usually a pronounced bradycardia (Table 2 and Figure 1) and occasionally ventricular ectopic beats were present. The bradycardia was presumably reflex in origin (from stimulation of carotid and aortic baroceptors) since it was not

Table 2 Cardiovascular effects of intravenous diethylcarbamazine citrate (DECC, 2.5–10.0 mg/kg) in anaesthetized cats (after the initial 0–10 seconds)

	Control	*Maximum change from control induced by DECC*		
		2.5	5.0	10.0 mg/kg
Systolic blood pressure (mmHg)	124 ± 4	+ 19 ± 6 (9)	+ 40 ± 6 (17)	+ 82 ± 6 (36)
Diastolic blood pressure (mmHg)	91 ± 4	+ 14 ± 3 (9)	+ 31 ± 4 (17)	+ 52 ± 4 (36)
Mean blood pressure (mmHg)	102 ± 4	+ 16 ± 4 (9)	+ 34 ± 5 (17)	+ 62 ± 5 (36)
Heart rate (beats/min)*	203 ± 8	+ 6 ± 2 (6) – 4 ± 1 (3)	– 18 ± 3 (8) + 7 ± 1 (5)	– 25 ± 5 (24) + 20 ± 5 (7)
Systolic ejection time (ms)	116 ± 8	– 2(1)	– 4 (1)	+ 12 ± 2 (9)
Left ventricular d*P*/d*t* *max.* (mmHg s^{-1})	3870 ± 800	+ 70 (1)	+ 640 ± 210 (3)	+ 2100 ± 140 (6)
LVEDP (mmHg)	3.4 ± 0.4	0 (1)	+ 0.5 (2)	+ 0.7 ± 0.3 (5)
Carotid artery d*P*/d*t* *max** (mmHg s^{-1})	2200 ± 120	– 180 ± 40 (7) + 261 (1)	– 430 ± 120 (8) + 410 ± 60 (5)	– 250 ± 140 (3) + 980 ± 120 (25)
Pulmonary artery pressure (systolic, mmHg)	19 ± 1	+ 2.0 ± 0.5 (8)	+ 5 ± 1.0 (8)	+ 17 ± 3 (9)
Pulmonary artery pressure (diastolic, mmHg)	11 ± 0.5	1.0 ± 0.2 (8)	+ 2 ± 0.5	+ 7.5 ± 2.0 (9)

The values are the mean changes from control (± s.e.); number of observations in parentheses.

* The heart rate and carotid artery d*P*/d*t* *max.* responses to DECC varied; in some increases occurred, in others there were decreases. These have been separated in the Table. The changes induced by DECC were significantly different ($P < 0.001$) from those induced by an equivalent volume of saline.

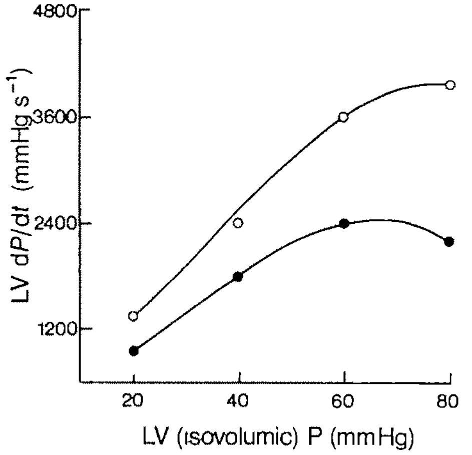


Figure 2 The effect of diethylcarbamazine citrate (DECC, 10 mg/kg i.v.) on the relationship between left ventricular d*P*/d*t* and left ventricular pressure during the period before the opening of the aortic valves (66 mmHg in the control situation). The higher ratio of d*P*/d*t* to peak common developed isovolumic pressure (CPIP, at 60 mmHg) after the administration of DECC indicates an increase in the contractile state of the ventricle. Control (●); DECC (○).

observed in cats subjected to bilateral vagotomy (Figure 1); under these conditions DECC increased heart rate. No ventricular arrhythmias were observed after vagotomy. There were indications that DECC increased myocardial contractility. Increases occurred in cardiac output (measured at the height of the pressure reponse) of 29 ml kg^{-1} min^{-1} (with a dose of 5 mg/kg DECC; single estimation) and of 73 ± 21 ml kg^{-1} min^{-1} with a dose of 10 mg/kg (3 estimations); the control cardiac output was 171 ± 6 ml kg^{-1} min^{-1}. Stroke volume was also increased (from 0.8 ± 0.04 ml/beat to 1.15 ± 0.15 ml/beat with a dose of 10 mg/kg). There was also substantial increases in pulse pressure, in carotid d*P*/d*t* *max.* and in left ventricular d*P*/d*t* *max.* (Table 2). Since there were also slight increases in LVEDP (Table 2) it is not possible to deduce with certainty from these results alone that a true increase in contractility had occurred. However it is doubtful whether an increase in LV d*P*/d*t* *max.* of 2100 ± 130 mmHg s^{-1} (from a pre-DECC level of 3870 ± 800 mmHg s^{-1} i.e. 55%) can be explained on the basis of the Frank-Starling mechanism alone, by an increase in ventricular filling pressure of only 0.7 mmHg (from 3.4 mmHg). Because of the marked changes in afterload, measurements were also made of d*P*/d*t* at fixed isovolumic left ventricular pressures before, and after, DECC. The results of one such experiment are illustrated in Figure 2. It is clear that the increases that occurred in d*P*/d*t* cannot be explained solely on the basis of changes in afterload.

Effects of diethycarbamazine citrate on myocardial blood flow The effect of DECC on myocardial temperature (measured with implanted heated thermocouples) was determined in four cats. The myocardial temperature was a mean of 0.2°C higher than that of the aortic blood and a constant heating current of 0.15 A^2 raised myocardial temperature by a further 0.90°C. Intravenous injections of DECC 5 mg/kg (3 injections) and 10 mg/kg (10 injections) decreased intramyocardial temperature by 0.075 ± 0.03°C and by 0.24 ± 0.03°C respectively; the peak change occurred 1–2 min after the injection and corresponded to the peak pressure response. The effect lasted for between 4 and 5 minutes.

Modification of the haemodynamic effects of diethylcarbamazine citrate by hexamethonium, phentolamine and bethanidine The secondary, hypertensive, effect of DECC was markedly reduced by the prior administration of hexamethonium (0.5 mg/kg intravenously; Figure 3). Thus in four control experiments DECC (10 mg/kg) increased systolic blood pressure by 97 ± 23 mmHg (from 109 ± 28 mmHg; mean ± s.e.), diastolic blood pressure by 62 ± 21 mmHg (from 78 ± 27 mmHg), heart rate by 17 ± 15 beats/min (from 178 ± 19 beats/min) and carotid d*P*/d*t* *max.* by 1440 ± 520 mmHg s^{-1} (from 1690 ± 140 mmHg s^{-1}). After the intravenous administration of hexamethonium the increases in pressure were only 15 ± 2 mmHg (from 77 ± 21 mmHg, systolic, and 13 ± 2 mmHg (from 51 ± 21 mmHg, diastolic); there was no change in heart rate and carotid d*P*/d*t* *max.* was actually reduced by a mean of 240 mmHg s^{-1} (from 1590 mmHg s^{-1}). Hexamethonium also prevented the DECC-induced changes in myocardial blood flow. Similar reductions in the secondary haemodynamic effects of DECC were also observed after the prior administration of bethanidine (3 mg/kg; 5 experiments, Table 3) and of phentolamine (0.5 mg/kg; 2 experiments). For example, in these 2 experiments, DECC raised systemic blood pressure by a mean of 65 mmHg (systolic) and by 53 mmHg (diastolic) and increased LV d*P*/d*t* *max.* by 1540 mmHg s^{-1} (77%) and heart rate by 9 beats/minute. The corresponding changes after phentolamine were 16 and 9 mmHg and + 60%; there was a decrease in heart rate of 30 beats/minute.

Effects of diethylcarbamazine citrate on intratracheal pressure; modification of response to prostaglandin $F_{2\alpha}$

DECC usually slightly increased intratracheal pressure at constant inflation volumes (i.e. it decreased pulmonary compliance); this effect was dose-related. From the pre-injection peak inspiratory pressure of 6.0 ± 0.4 mmHg the changes, following the administration of DECC (2.5, 5 and 10 mg/kg), were

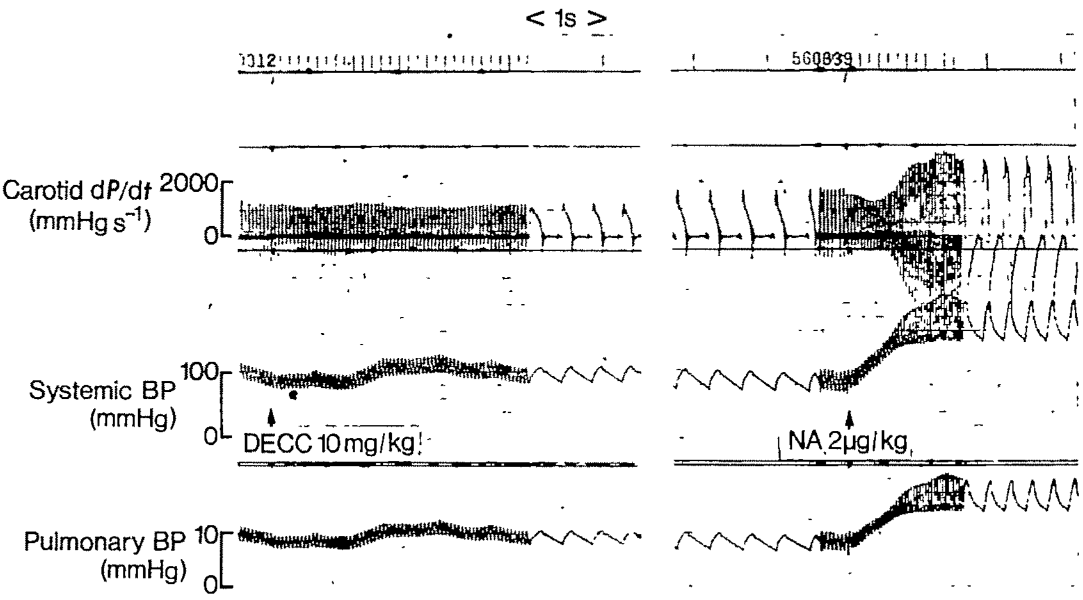


Figure 3 Cardiovascular effects of diethylcarbamazine citrate (DECC, 10 mg/kg i.v.) in an anaesthetized cat after the administration of hexamethonium (0.5 mg/kg). The systemic and pulmonary vascular effects (Figure 1) were almost completely inhibited, responses to noradrenaline (NA, 2 µg/kg i.v.) were unaffected.

+ 0.2 ± 0.1 mmHg (+ 3.3%), + 0.3 ± 0.1 mmHg (+ 5%) and + 0.7 ± 0.1 mmHg respectively (7 observations at each dose level). It is possible that these changes were mediated through reflex vagal activity. In view of the observation that DECC antagonizes the effect of prostaglandins E_2 and $F_{2\alpha}$ on isolated bovine pulmonary venous strips (Burka & Eyre, 1974), and in view of the claimed efficacy of DECC in asthma (for references see introductory section), experiments were performed to investigate the effect of the drug on prostaglandin-induced changes in pulmonary compliance. A single dose of prostaglandin $F_{2\alpha}$ was chosen (2.5 µg/kg, i.v.) and this increased intratracheal pressure by 2.4 ± 0.3 mmHg (from the control pressure of 7.8 ± 0.4 mmHg (i.e. by 30%); increases in mean pulmonary artery pressure (from 16 ± 1 to 26 ± 1 mmHg) were also observed with this dose of prostaglandin F_2 (Figure 4). DECC, in doses up to 10 mg/kg administered intravenously 30 s before prostaglandin $F_{2\alpha}$, had no effect on this pulmonary vascular response (16 ± 1 mmHg before prostaglandin $F_{2\alpha}$ and 28 ± 1 mmHg after) but, at the higher dose levels (5 and 10 mg/kg) it did reduce the prostaglandin-induced increase in intratracheal

Table 3 Cardiovascular effects of diethylcarbamazine citrate (DECC, 5 and 10 mg/kg i.v.) before, and 1 h after, the intravenous administration of bethanidine (3 mg/kg)

	Before bethanidine			*After bethanidine*		
	Control	*Change induced by DECC*		*Control*	*Change induced by DECC*	
		5 mg/kg	*10 mg/kg*		*5 mg/kg*	*10 mg/kg*
Systolic blood pressure (mmHg)	115 ± 15	+ 50 ± 5	+ 130 ± 20	97 ± 10	+ 19 ± 5*	+ 33 ± 13*
Diastolic blood pressure (mmHg)	88 ± 14	+ 48 ± 6	+ 96 ± 14	59 ± 9	+ 17 ± 5*	+ 29 ± 10*
Heart rate (beats/min)	207 ± 23	+ 6 ± 4	+ 29 ± 27	221 ± 17	− 5 ± 2	− 6 ± 13
Pulmonary artery pressure	16 ± 1	+ 3.2 ± 0.7	+ 13 ± 4.1	17 ± 1	+ 1.7 ± 0.5	+ 3.0 ± 1.2**

Values are means of five experiments ± s.e. of the mean.
* $P < 0.01$; ** $P < 0.05$ compared with change before bethanidine.

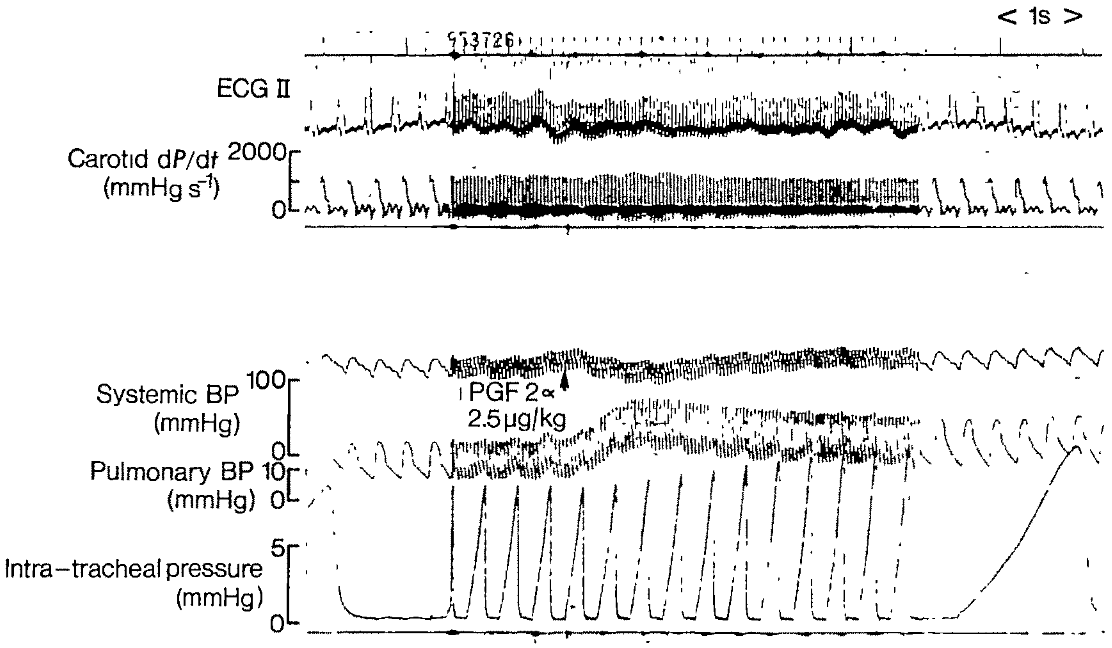


Figure 4 The effects of an intravenous injection of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 2 5 μg/kg) on the E C G, carotid arterial d*P*/d*t* and pressure, pulmonary artery pressure and intra-tracheal pressure, of a cat anaesthetized with pentobarbitone The most pronounced effects of prostaglandin $F_{2\alpha}$ were increases in pulmonary artery pressure and in intra-tracheal pressure (from 8.8 to 11.6 mmHg at the end of inspiration, i e +32%)

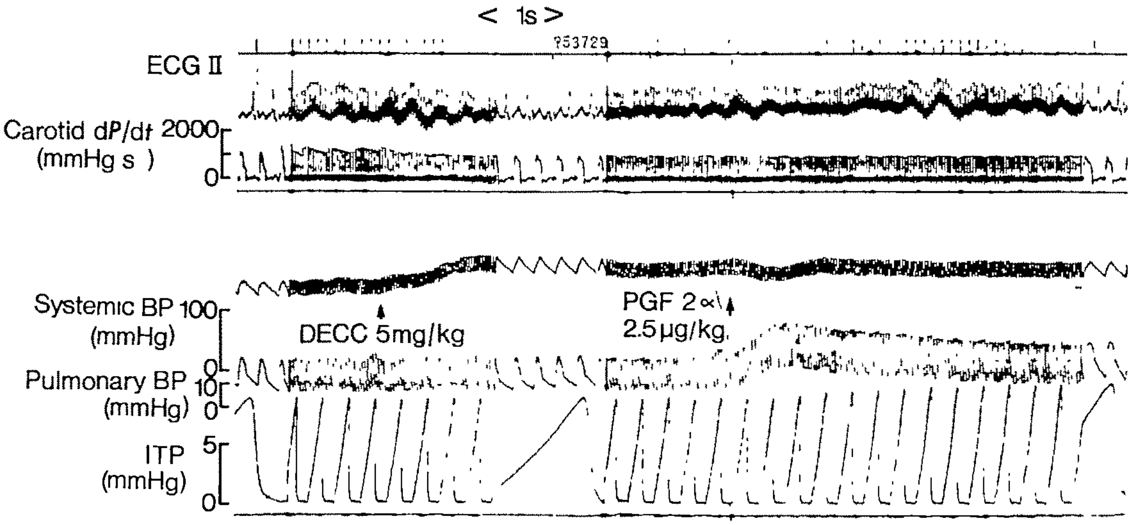


Figure 5 Same experiment as Figure 4 Modification of the effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on intratracheal pressure by the prior administration of diethylcarbamazine citrate (DECC, 5 mg/kg). The increase in intratracheal pressure (ITP) was from 8.8 to 10 0 mmHg at the end of inspiration (i e. +14%) compared with +32% in the control situation (Figure 4).

pressure (Figure 5). Thus the change after 5 mg/kg DECC was 1.4 ± 0.3 mmHg ($P < 0.05$ compared with the control prostaglandin F_2 response of 2.4 ± 0.4 mmHg; 5 experiments) and after 10 mg/kg DECC the increase was only 0.7 mmHg (2 experiments).

Effects of diethylcarbamazine citrate on the nictitating membrane In two cats the intravenous administration of DECC (5 mg/kg, 3 experiments and 10 mg/kg, 15 experiments) resulted in contractions of the nictitating membrane with no marked effect on the response to pre-ganglionic nerve stimulation. In a third cat, similar doses of DECC did not contract the membrane (4 experiments) although they did potentiate the response to nerve stimulation, an effect that was not observed with a dose of DMPP (30 µg/kg, i.v.) that caused a similar, marked increase in systemic arterial pressure. The effects of DECC on the nictitating membrane were prevented by the prior administration of hexamethonium (0.5 mg/kg) or phentolamine (0.5 mg/kg).

Isolated coronary arteries

In view of the increases in local myocardial blood flow observed when DECC was administered to anaesthetized cats, experiments were performed to see whether the drug relaxed isolated coronary arteries *in vitro*. In concentrations of 2.6×10^{-2} M to 2.0 mM, DECC caused dose-dependent relaxations of pig coronary artery strips contracted with potassium (10 to 20 mM); the ED_{50} was 2.0 mM (compared with isoprenaline on the same preparations, $ED_{50} = 5 \times 10^{-5}$ mM; 11 estimations). The DECC response was unaffected by propranolol in a dose (1.7×10^{-3} mM) that prevented the response to isoprenaline.

Discussion

These studies demonstrate that the intravenous administration of DECC in anaesthetized cats gives rise to cardiovascular changes strikingly similar to those that result from the administration of nicotine. Commencing 5–10 s after the injection, there were pronounced increases in both systemic and pulmonary arterial pressures and there was a contraction of the nictitating membrane. There was also evidence (increases in cardiac output and in dP/dt at fixed isovolumic pressures) of a positive inotropic effect on the heart. These changes were associated with an increase in myocardial blood flow, an effect presumably secondary to the increases in perfusion pressure and in cardiac work; only in high concentrations does DECC relax coronary arteries *in vitro*. Since the hypertensive effect was markedly reduced by phentolamine, and since both the systemic hypertension and the increases in myocardial contractility and blood flow were reduced by hexamethonium and by bethanidine, it seems reasonable to conclude that these effects of DECC are secondary to stimulation of sympathetic ganglia. It is doubtful whether direct stimulation of the adrenal medulla contributes significantly to these haemodynamic effects since, in studies designed to examine the effect of DECC on the soleus and tibialis muscles of the cat, Abaitey & Nott (unpublished) found that the pressure response to DECC was unaffected by removal of the adrenal glands. The cardiovascular changes were sometimes accompanied by ventricular premature beats, particularly at the height of the pressure response. This is rather similar to the effect obtained when noradrenaline is infused intravenously (particularly in the presence of myocardial ischaemia; Marshall & Parratt, 1973) and was not observed after bilateral vagotomy. Ventricular premature beats, as well as irregular sinus rhythm and periods of sinus arrest were also reported following the rapid intravenous administration of DECC in conscious dogs by Harned *et al.* (1948).

A further point of similarity between DECC and nicotine was the initial bradycardia and the reductions in systemic and pulmonary artery pressures and in left ventricular and carotid artery dP/dt *max.* These effects occurred within the initial 4–5 s of an intravenous injection and were more pronounced, and more rapid in onset, following injection into the pulmonary artery. In close-chest, spontaneously breathing, cats there was also inhibition of respiration lasting 7–10 seconds. This combination of apnoea, bradycardia and hypotension is similar to the response to the intravenous administration of diguanides and isothiourea derivatives (Dawes & Fastier, 1950), of 5-hydroxytryptamine (Comroe, Van Lingen, Stroud & Roncoroni, 1953) and of nicotine itself (Armitage & Hall, 1969) and the fact that the effects were prevented by bilateral vagotomy suggests that DECC stimulates vagal receptors in the pulmonary vascular bed. When DECC was injected directly into the lumen of the left ventricle there was a slight decrease in left ventricular dP/dt *max.* but almost no systemic hypotension. This may suggest that DECC does not stimulate receptors in the coronary circulation (the 'coronary chemoreflex' described by Dawes & Comroe, 1954). It is clear that receptors situated at the endocardial surface of the left ventricle are also not stimulated by the drug; no attempt was made to stimulate epicardial receptors (Sleight, 1964) by instilling DECC into the pericardial sac.

Among the adverse reactions attributed to DECC are headache and, in severely debilitated patients, a weak pulse, respiratory distress and coma (for references see introductory section). The headaches may be associated with increased systemic pressure. To our knowledge, blood pressure has not been measured in man following the intramuscular or oral administration of DECC but the recommended clinical dose (6–16 mg/kg in single or divided doses in the treatment of *Wuchereria bancrofti* and *Loa loa* and 10 mg/kg in the treatment of asthma) is clearly within the range of that used in the present studies. There was no evidence for a direct depressant effect of DECC on the myocardium, even when injected in a high concentration directly into the lumen of the left ventricle. However, it must be recognized that these were essentially normal cats and it would not be valid to draw the conclusion from these experiments alone that DECC has no depressant effect on the myocardium already damaged by ischaemia or disease.

The use of DECC in intractable asthma (i.e. asthma usually unresponsive to standard bronchodilators) was first suggested by Mallen in 1965. He observed excellent results in an asthmatic patient who was given DECC to treat tropical eosinophilia. This observation led to its successful use in other asthmatic patients (Mallen, 1965; Srinivas & Antani, 1971) and, more recently, it has been demonstrated that DECC protects against exercise-induced airway obstruction in asthmatic children (Sly & Matzen, 1974). DECC is itself without bronchodilator activity (Sly & Matzen, 1974 and present paper). One possible explanation for the apparent effectiveness of DECC in asthma is its ability to inhibit the release of slow reacting substance-A (Orange, Valentine & Austen, 1968) but the recent observation by Burka & Eyre (1974) that it antagonizes prostaglandin $F_{2\alpha}$-induced contractions of bovine pulmonary veins, prompted us to examine its effect against the pulmonary responses to this prostaglandin *in vivo*. When given just before prostaglandin F_2 , DECC reduced the changes that occurred in pulmonary compliance without influencing the pulmonary vascular response. This does not necessarily imply the presence of two receptor types for prostaglandin F_2; DECC-induced release of catecholamines would be another, simpler explanation. Clearly, however, this is a promising area for further experimental study.

A.K.A. is a Commonwealth scholar on leave from the Faculty of Pharmacy, University of Science and Technology, Kumasi, Ghana. We are grateful to Dr A.F. Green (Wellcome Research Labs, Beckenham, Kent) for generous supplies of diethylcarbamazine citrate and to Dr J.E. Pike (Upjohn Co., Kalamazoo, U.S.A.) for prostaglandin F_2 .

References

ARMITAGE, A.K. & HALL, G.H. (1969). Mode of action of intravenous nicotine in causing a fall of blood pressure in the cat. *Eur. J. Pharmac.*, **7**, 23–30.

BURKA, J.F. & EYRE, P (1974). Studies of prostaglandins and prostaglandin antagonists on bovine pulmonary vein *in vitro*. *Prostaglandins*, **6**, 333–343.

COMROE, J.M., VAN LINGEN, B., STROUD, R.C. & RONCORONI, A. (1953). Reflex and direct cardiopulmonary effects of 5-OH tryptamine (Serotonin). *Amer. J. Physiol.*, **173**, 379–386.

DAWES, G.S. & COMROE, J.H (1954). Chemoreflexes from the heart and lungs. *Physiol. Rev.*, **34**, 167–201.

DAWES, G.S. & FASTIER, F.N. (1950). Reflex actions of some isothiourea derivatives on circulation and respiration. *Br. J. Pharmac.*, **5**, 323–334.

FORBES, L.S. (1972). Pharmacodynamic actions of diethylcarbamazine in the dog with particular reference to nicotine-like properties. *S.E. Asian J. Trop. Med.* **3**, 99–102.

FUGLSANG, J. & ANDERSON, J. (1974). Collapse during treatment of onchocerciasis with diethylcarbamazine. *Trans. Roy. Soc. Trop. Med. Hyg.*, **86**, 72–73.

GOLDSMITH, R.S (1972). Anthelmintic drugs. In *Review of Medical Pharmacology*, Ed. Meyers, F.H., Jawetz, E. & Goldfien, A. pp. 594–619. Los Altos: Lange Medical.

HARNED, B.K., CUNNINGHAM, R.W., HALLIDAY, S., VESSEY, R.E., YUDA, N.N., CLARK, M.C. & SUBBA ROW, Y. (1948). Some toxicological and pharmacological properties of 1-diethylcarbamyl-4-methylpiperazine hydrochloride, hetrazan. *Ann. N.Y. Acad. Sci.*, **50**, 141–160.

KOIVIKKO, A. (1973). Diethylcabamazine in bronchial asthma. *Ann. Allergy*, **31**, 45–46.

MALLEN, M.S. (1965). Treatment of intractable asthma with diethylcarbamazine citrate. *Ann. Allergy*, **23**, 534–537.

MARSHALL, R J. & PARRATT, J.R. (1973). The effect of noradrenaline on blood flow and oxygen consumption in normal and ischaemic areas of myocardium. *Amer. Heart J.*, **86**, 653–662.

MASON, D.T. (1969). Usefulness and limitations of the rate of rise of intraventricular pressure (d*P*/d*t*) in the evaluation of myocardial contractility in man. *Amer. J. Cardiol.*, **23**, 516–527.

OOMEN, A.P (1969). Fatalities after treatment of onchocerciasis with diethylcarbamazine. *Trans. Roy. Soc. Trop. Med. Hyg.*, **63**, 548.

ORANGE, R.P., VALENTINE, M.D. & AUSTEN, K.F. (1968). Inhibition of the release of slow-reacting substance of anaphylaxis in the rat with diethylcarbamazine. *Proc. Soc. exp. Biol. Med.*, **27**, 127–132.

PARRATT, J.R. (1973). Myocardial and circulatory effects of *E. coli* endotoxin; modification of responses to catecholamines. *Br. J. Pharmac.*, **47**, 12–25.

PARRATT, J.R. & WADSWORTH, R.M. (1972). The effects of dipyridamole on coronary post-occlusion hyperaemia and on myocardial vasodilatation induced by systemic hypoxia. *Br. J. Pharmac.*, **46**, 594–601.

SLEIGHT, P. (1964). A cardiovascular depressor reflex from the epicardium of the left ventricle in the dog. *J. Physiol.*, **173**, 321–343.

SLY, R.M. & MATZEN, K. (1974). Effect of diethylcarbamazine pamoate upon exercise-induced obstruction in asthmatic children. *Ann. Allergy*, **33**, 138–144.

SRINIVAS, H.V. & ANTANI, J. (1971). Diethylcarbamazine in bronchial asthma. *Ann. Allergy*, **29**, 418–421.

(Received September 1, 1975)

Br. J. Pharmac. (1976) **56**, 229–233

INFLAMMATORY EFFECTS OF PROSTAGLANDIN D_2 IN RAT AND HUMAN SKIN

R.J. FLOWER, E A. HARVEY & W.P. KINGSTON
Department of Pharmacology, Wellcome Research Laboratories, Beckenham, Kent

1 Intradermal injection of prostaglandin (PG) D_1 and D_2 in the human forearm produced a long-lasting dose-related erythema. When compared with prostaglandin E_1 or E_2 the order of potency for erythema production was $PGE_1 > PGE_2 > PGD_2 > PGD_1$.

2 In rat skin, prostaglandin D_2 but not D_1 caused an increase in vascular permeability as quantitated by the Evans blue method and the ^{125}I-albumin extravasation technique. Prostaglandin E_2 was 3–5 times more potent than prostaglandin D_2.

3 Prostaglandin D_2 (10 ng) potentiated the increase in vascular permeability in rat skin produced by histamine, but not that produced by bradykinin.

4 Prostaglandin D_2 (10, 20 and 50 ng) did not elicit oedema or hyperalgesia in the rat paw oedema test, but potentiated carrageenan-induced oedema; hyperalgesia was potentiated by doses of 100 ng and above.

Introduction

There is increasing evidence for the involvement of prostaglandins of the E and F series in the inflammatory process. Whereas the E prostaglandins are pro-inflammatory, the F prostaglandins have anti-inflammatory properties.

Intradermal injections of prostaglandin E_1 produce a long-lasting erythema (Solomon, Juhlin & Kirschbaum, 1968; Juhlin & Michaelson, 1969) and hyperalgesia (Ferreira, 1972) and potentiate the increase in vascular permeability (Williams & Morley, 1973; Moncada, Ferreira & Vane, 1973; Thomas & West, 1974), pain (Ferreira, 1972) and itching (Greaves & McDonald-Gibson, 1973) produced by bradykinin or histamine. Increased amounts of E prostaglandin are found in inflamed skin (Sondergaard & Greaves, 1970; Greaves, Sondergaard & McDonald-Gibson, 1971; Angaard & Jonsson, 1971; Hamberg & Jonsson, 1973), in ocular (Eakins, Whitelocke, Perkins, Bennet & Ungar, 1972) and in many other types of inflammation (Ferreira, Flower, Moncada & Vane, 1975).

Prostaglandins of the F series antagonize some effects of the E prostaglandins (Crunkhorn & Willis; 1971). Prostaglandin $F_{2\alpha}$ is a veno-constrictor (Sweet, Kadowitz & Brody, 1971) and is found in increased amounts in the later stages of inflammation (Velo, Dunn, Giroud, Timsit & Willoughby, 1973), suggesting that it is involved in the termination of the inflammatory reaction.

The 'endoperoxide' intermediate (prostaglandin H_2) in prostaglandin biosynthesis may break down to form prostaglandin D in addition to prostaglandins E and F (Granström, Lands & Samuelsson, 1968) and formation of all three prostaglandins by skin homogenates has been observed (Nugteren & Hazelhof, 1973; Kingston, 1975, unpublished observation). In view of the involvement of E and F prostaglandins in the inflammatory process, we have also studied the action of prostaglandins D_1 and D_2.

Methods

Intradermal injections in man and measurements of erythema

Double blind studies were carried out in 5 male volunteers who gave their informed consent. Prostaglandin solutions (in pyrogen-free sterile 0.9% w/v NaCl solution, saline) were injected intradermally in a volume of 0.1 ml into the volar surface of the human forearm.

Erythema was measured 30 min after injection by covering the skin with a clear plastic sheet and tracing the contours of the intense erythema on to the plastic. A photocopy of the area of response was made and the area representing erythema was cut out and weighed.

Measurement of vascular permeability changes in rat skin

Prostaglandins D_1, D_2, E_1, E_2, bradykinin or histamine were injected (in 0.1 ml sterile saline) intradermally

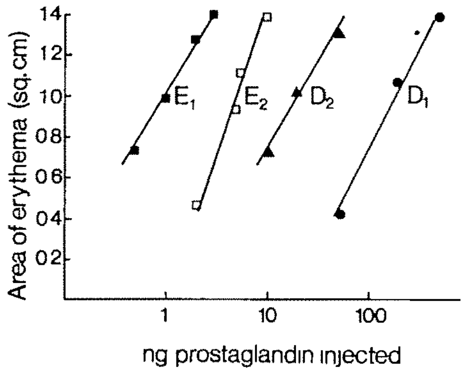


Figure 1 Dose related erythema in the human forearm 30 min after intradermal injection of prostaglandin E_1 (■), prostaglandin E_2 (□), prostaglandin D_2 (▲), prostaglandin D_1 (●).

either alone or as a mixture into the (shaved) abdominal skin of Olac rats (150–200 g). Control injections of saline were also made in each animal. The effects on vascular permeability were quantitated by the Evans blue method and by measurement of extravasation of ^{125}I-albumin.

Evans blue elution

Five minutes prior to intradermal injection, each rat was injected intravenously with Evans blue (40 mg/kg). Rats were killed 30 min after intradermal injection. The skin was removed and discs of skin (which included the whole of the lesion) were excised with a 15 mm punch. The skin was frozen in liquid nitrogen and disintegrated by hammering in a cooled stainless steel mortar. The Evans blue was then extracted in 4 ml of formamide and the absorbance at 600 nm measured, as described by Rees, Okino & Rocha e Silva (1971).

Measurement of extravasation of ^{125}I-albumin

Immediately prior to intradermal injection of inflammatory agents 5 µCi of ^{125}I-albumin was injected into a tail vein. Evans blue (40 mg/kg) was also injected to visualize the sites of increased vascular permeability. After 20 min the animals were killed and the blue areas excised with a punch as already described. The excised skin was then wrapped in a single layer of 'Parafilm' and the radioactivity measured in a gamma counter. The radioactivity in 10 µl of blood was measured as a reference.

Measurement of carrageenan-induced oedema and hyperalgesia in the rat paw

Oedema and hyperalgesia were measured in male Olac rats (130–150 g) after injection of either carrageenan or prostaglandin D_2 alone, and the results compared with those obtained with a mixture of carrageenan and prostaglandin D_2. The rats were treated with indomethacin (10 mg/kg) 30 min prior to carrageenan injection to abolish endogenous prostaglandin release. Injections (0.1 ml) of prostaglandin E_2, D_2 and/or carrageenan (0.5% in saline) were made into one of the hind paws of a rat. The contralateral (control) paw was injected with 0.1 ml saline. The oedema was determined by mercury displacement manometry. Hyperalgesia was measured by applying an increasing pressure to the paw and measuring the time taken for the animal to react by withdrawal of the paw (Randal & Selitto, 1957). The oedema and hyperalgesia elicited by inflammatory agents was in each case compared with that produced by saline in the contralateral paw.

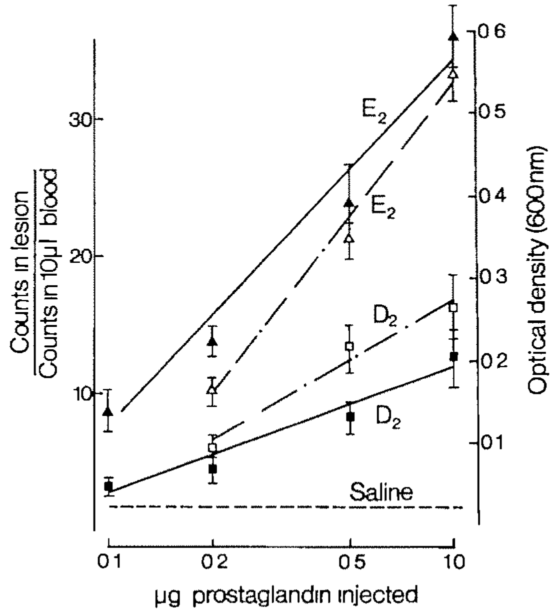


Figure 2 Increased vascular permeability elicited by intradermal injection of Prostaglandin E_2 or D_2 in rat skin as measured by 2 assay methods·

(a) Evans blue elution (——·——, right ordinate) from lesions produced by intradermal injection of prostaglandin E_2 (△), prostaglandin D_2 (□)

(b) Extravasation of ^{125}I-albumin (——, left ordinate) elicited by intradermal injection of prostaglandin E_2 (▲), prostaglandin D_2 (■)

Results expressed as mean ± s.e. mean. (– – – –), represents effect of intradermal injection of saline (0 1 ml)

Materials

Evans blue was obtained from Phase Separations; ^{125}I-albumin from the Radiochemical Centre, Amersham;

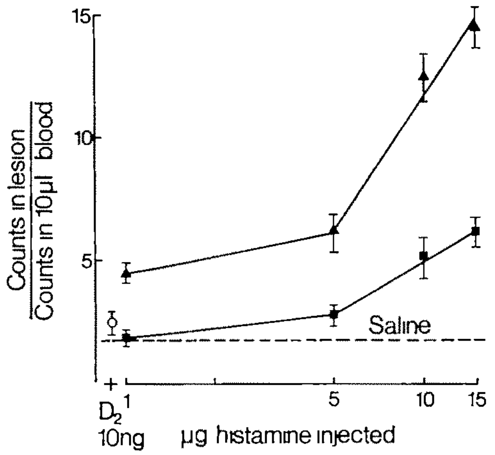


Figure 3 Effect of prostaglandin D_2 on the ^{125}I-albumin extravasation in rat skin elicited by histamine (■), 10 ng prostaglandin D_2 (O), histamine+10 ng prostaglandin D_2 (▲). Results are expressed mean ± s e. mean (———), represents effect of intradermal injection of saline (0.1 ml).

bradykinin from Schwarz/Mann; histamine acid phosphate from B.D.H.; sodium carrageenan from Marine Colloids; and indomethacin from Merck, Sharpe & Dohme. Prostaglandins E_1, E_2, $F_{2\alpha}$, D_1 and D_2 were pure as judged by thin layer chromatography.

Results

Effects of intradermal injection of D prostaglandin in man

In human skin, intradermal prostaglandin D_1 or D_2 produced long-lasting erythema with relatively little oedema. The erythema was dose-related and was maximal some 30 min after injection. For prostaglandin D_1, the threshold dose was about 50 ng, and for prostaglandin D_2 it was 2–10 ng. Increased vasodilatation was still visible 4 h after injection of 500 ng prostaglandin D_1 and 6 h after the same dose of prostaglandin D_2.

The results obtained for prostaglandins E_1, E_2, D_1 and D_2 were qualitatively similar in each trial but there was considerable inter-subject variation in the size of erythema elicited by a given dose of prostaglandin. For this reason the results in an individual subject are shown in Figure 1. Prostaglandin E_1, E_2, D_2 and D_1 produced fairly parallel dose response curves. The potency ratio E_1:E_2:D_2:D_1, was 1:5:25:300. No hyperalgesia was evident at the sites of prostaglandin D_1 or D_2 injection up to doses of 500 ng.

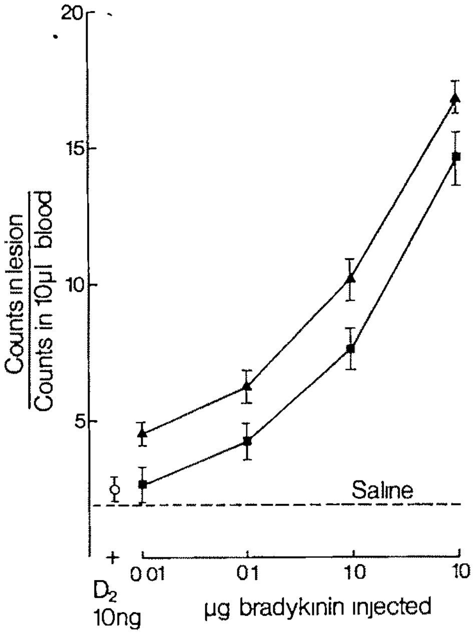


Figure 4 Effect of 10 ng prostaglandin D_2 on the increased vascular permeability in rat skin produced by intradermally injected bradykinin Results are expressed (mean ± s e mean). Effects of bradykinin (■), 10 ng prostaglandin D_2 (O), bradykinin+10 ng prostaglandin D_2 (▲). Bradykinin solution contained 2 µg of bradykinin potentiating peptide 5A. (———), represents effect of intradermal injection of saline (0 1 ml)

Effects of D prostaglandins on rat skin

Intradermal injection of prostaglandin D_2 into rat skin produced an increase in vascular permeability with doses as low as 10 ng. Prostaglandin D_1 at doses up to 10 µg did not increase vascular permeability. The dose response curves for prostaglandins E_2 and D_2 on vascular permeability were not parallel. Prostaglandin E_2 was 3–5 times more potent than prostaglandin D_2. Both the Evans blue method and the ^{125}I-albumin method gave similar results (Figure 2).

Actions of PGD$_2$ on cutaneous responses to histamine and bradykinin in the rat

In the rat skin, the increase in vascular permeability induced by histamine was potentiated, in excess of simple summation, by simultaneous administration of 10 ng of prostaglandin D_2 (Figure 3). Prostaglandin D_2 (10–100 ng) also slightly enhanced the increased permeability produced by bradykinin (Figure 4), but this enhancement could have been due to simple

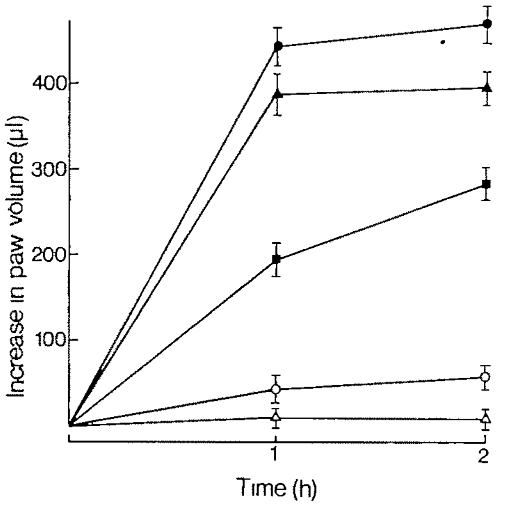


Figure 5 Potentiation by prostaglandins E_2 and D_2 of the rat paw swelling induced by carrageenan Increase in paw volume (mean ± s.e. mean) elicited by 0 5% carrageenan (■), 0.5 ng prostaglandin E_2 (○), carrageenan + 0.5 ng prostaglandin E_2 (●), 10 ng prostaglandin D_2 (△), carrageenan + 10 ng prostaglandin D_2 (▲). In each case the oedema produced by the test agent was calculated by subtraction of the effect of saline injection in the contralateral paw.

summation of the effects of prostaglandin D_2 and bradykinin.

Action of prostaglandin D_2 on the carrageenan-induced oedema and hyperalgesia in the rat paw

Injection of 10, 20 and 50 ng prostaglandin D_2 alone into the rat paw produced neither oedema nor hyperalgesia. Oedema produced by carrageenan was potentiated by about 100% at 1 h after simultaneous injection of 10 ng prostaglandin D_2 (Figure 5 and Table 1). A similar effect was produced by 0.5 ng prostaglandin E_2. Prostaglandin D_2 (100 ng) elicited a slight hyperalgesia, and potentiated carrageenan induced hyperalgesia by about 100% at 1 h (Table 1).

Discussion

E prostaglandins elicit inflammatory responses in the skin including vasodilatation (Juhlin & Michaelsson, 1969; Solomon, Juhlin & Kirschbaum, 1968) increased vascular permeability (Kaley & Weiner, 1971) and hyperalgesia (Ferreira, 1972). Two important characteristics of the inflammatory properties of E prostaglandins are the long duration of action (Juhlin & Michaelsson, 1969; Ferreira, 1972), and their ability to potentiate the effects of other mediators of inflammation (Ferreira, 1972; Williams & Morley, 1973; Ferreira, Moncada & Vane, 1973; Greaves & McDonald-Gibson, 1973; Thomas & West, 1974). We have now shown that the inflammatory effects of prostaglandin D_2 show the same two characteristics. Both prostaglandins D_1 and D_2 produce a long-lasting erythema in the human skin. Like the E prostaglandins (Juhlin & Michaelsson, 1969; Ferreira, 1972) they produce vasodilatation more effectively than oedema. It is of interest that for the production of erythema, prostaglandin E_1 is more potent than E_2, whereas prostaglandin D_1 is less potent than D_2. No hyperalgesia was noted at the sites of intradermal injections of up to 500 ng of prostaglandins D_1 or D_2. In addition to eliciting the inflammatory response when given alone, prostaglandins D_2 and E_2 potentiate (in much lower concentrations) the inflammatory effects of histamine and carrageenan. Thus it appears that these prostaglandins could promote inflammation at two levels: at low concentrations they potentiate the effects of other inflammatory mediators, and at higher concentrations they additionally produce direct inflammatory effects. The importance of the potentiating ability of these prostaglandins is highlighted by the fact that even very low concentrations increase by 100% the vascular permeability produced by histamine or carrageenan.

Unlike prostaglandin E_2, prostaglandin D_2 does not potentiate the increased vascular permeability induced by bradykinin. This suggests an important difference in the inflammatory actions of the two prostaglandins. Since the peak of histamine production precedes that of bradykinin in the inflammatory reaction (Di Rosa, Giroud & Willoughby, 1971),

Table 1 Threshold doses of prostaglandins D_2 and E_2 for production of oedema and hyperalgesia in the rat paw

Threshold dose	*PGE_2 (ng)*	*PGD_2 (ng)*	*Dose Ratio E_2·D_2*
For oedema alone	5.0	100	1·20
For potentiation of carrageenan-induced oedema	0 5	10	1:20
For hyperalgesia alone	10	100	1 10
For potentiation of carrageenan-induced hyperalgesia	10	100	1:10

prostaglandin D_2 could be important in modulating the effects of histamine in the early stage.

The amounts of prostaglandin D_2 or E_2 which potentiated the carrageenan-induced hyperalgesia in the rat paw were similar to those which produced a direct effect on cutaneous vasculature, and rather higher than those needed to potentiate the oedema.

Prostaglandin D_2 was in all its actions less potent than prostaglandin E_2. It would be of interest to determine the factors involved *in vivo* in the selective breakdown of the endoperoxide intermediate to prostaglandin D, E or F. It is possible that altering the ratio of prostaglandins exerts a fine control of the inflammatory process. Nugteren & Hazelhof (1973) reported that the formation of prostaglandin D is stimulated by a factor present in the cytosol and that in many tissues D prostaglandins are the main products of the endoperoxide breakdown. These authors also suggested that the D prostaglandins were devoid of biological activity. However, prostaglandin D_2 potently inhibits platelet aggregation (Mills & MacFarlane, 1974) and acts on a number of smooth muscle preparations (Horton & Hones, 1974; Hamberg, Hedqvist, Strandberg, Svensson & Samuelsson, 1975).

The presence of prostaglandin D_2 in inflammatory exudates has not yet been demonstrated and awaits the development of a sensitive assay for prostaglandin D_2.

We conclude that prostaglandin D_2 appears to possess similar inflammatory properties to the E prostaglandins, but is less potent, and should be considered as a potential mediator of the inflammatory reaction.

References

ÄNGAÅRD, E. & JONSSON, C.E (1971). Efflux of prostaglandins in lymph from scalded tissue. *Acta physiol. scand.*, **81**, 440–447

CRUNKHORN, P & WILLIS, A.L. (1971). Interaction between prostaglandins E and F given intradermally in the rat. *Br. J Pharmac.*, **41**, 507–512.

DI ROSA, M., GIROUD, J.P. & WILLOUGHBY, D.A. (1971). Studies of the mediators of acute inflammatory response induced in rats in different sites by carrageenan and turpentine. *J. Path.*, **104**, 13.

EAKINS, K.E., WHITELOCKE, R A.F., PERKINS, E.S., BENNET, A. & UNGAR, W.G. (1972). Release of prostaglandins in ocular inflammation in the rabbit *Nature, New Biol.*, **239**, 248–249.

FERREIRA, S.H. (1972) Prostaglandins, aspirin like drugs and analgesia. *Nature, New Biol.*, **240**, 200–203.

FERREIRA, S H., FLOWER, R.J, MONCADA, S. & VANE, J R. (1975). Inhibition of prostaglandin biosynthesis as the mechanism of action of non-steroid anti-inflammatory drugs. *Exerpta Medica* (In press).

GRANSTRÖM, E. LANDS, W.E.M. & SAMUELSSON, B. (1968). Biosynthesis of 9α, 15-dihydroxy-11-ketoprost-13-enoic acid. *J. biol. Chem.*, **243**, 4104–4108.

GREAVES, M.W. & McDONALD-GIBSON, W. (1973) Itch: Role of prostaglandins. *Brit. Med. J.*, **3**, 608–609.

GREAVES, M.W, SÖNDERGAARD, J & McDONALD-GIBSON, W. (1971). Recovery of prostaglandins in human cutaneous inflammation. *Brit. Med. J.*, **2**, 258–260.

HAMBERG, M., HEDQVIST, P., STRANDBERG, K., SVENSSON, J & SAMUELSSON, B. (1975). Prostaglandin endoperoxides iv. Effects on smooth muscle. *Life Sci.*, **16**, 451–462.

HAMBERG, M. & JONSSON, C.E. (1973). Increased synthesis of prostaglandins in the guinea pig following scalding injury. *Acta physiol. scand.*, **87**, 240–245.

HORTON, E.W & JONES, R.L. (1974). Biological activity of prostaglandin D_2 on smooth muscle. *Br. J. Pharmac.*, **52**, 110–111P.

JUHLIN, S. & MICHAELSSON, G. (1969). Cutaneous vascular reactions to prostaglandins in healthy subjects and in patients with urticaria and atopic dermatitis. *Acta Derm. Vener. (Stockholm)*, **49**, 251–261.

KALEY, G.D. & WEINER, R (1971). Prostaglandin E_1, a potential mediator of the inflammatory response. *Ann. N.Y. Acad. Sci.*, **180**, 338–350.

MILLS, D.C.B. & MacFARLANE, D.E (1974). Stimulation of human platelet adenyl cyclase by prostaglandin D_2. *Thrombosis Research*, **5**, 401–412.

MONCADA, S, FERREIRA, S.H. & VANE, J.R. (1973). Prostaglandins, aspirin like drugs and the oedema of inflammation. *Nature, Lond.*, **246**, 217–218.

NUGTEREN, D H & HAZELHOF, E (1973). Isolation and properties of intermediates in prostaglandin biosynthesis. *Bioch. Bioph. Acta*, **326**, 448–461.

RANDAL, L.O. & SELLITO, J J. (1957). A method for measurement of analgesic activity on inflamed tissue. *Arch. Int. Pharmacodyn.*, **61**, 409–419.

REES, M.W, OKINO, L & ROCHA e SILVA, M. (1971). Comparative pharmacological actions of bradykinin and related kinins of larger molecular weight. *Biochem. Pharmac.*, **20**, 2935–2946.

SÖNDERGAARD, J & GREAVES, M.W (1970). Pharmacological studies in inflammation due to exposure to ultraviolet radiation. *J. Path.*, **101**, 93–97.

SOLOMON, L M., JUHLIN, L & KIRSCHBAUM, M.M (1968). Prostaglandins on cutaneous vasculature. *J. Invest. Derm.*, **51**, 280–282.

SWEET, C.S, KADOWITZ, P J. & BRODY, M.J. (1971). A hypertensive response to infusion of prostaglandin $F_{2\alpha}$ into the vertebral artery of the conscious dog. *Eur. J. Pharmac.*, **16**, 229–232.

THOMAS, G. & WEST, G.B. (1974). Prostaglandins, Kinin and inflammation in the rat. *Br. J. Pharmac.*, **50**, 231–235.

VELO, G.P., DUNN, C.J., GIROUD, J.P., TIMSIT, J.& WILLOUGHBY, D.A (1973). Distribution of prostaglandins in inflammatory exudate. *J. Path.*, **111**, 149–158.

WILLIAMS, T.J. & MORLEY, H (1973). Prostaglandins as potentiators of increased vascular permeability in inflammation. *Nature, Lond.*, **246**, 215–217.

(Received September 4, 1975.
Revised October 14, 1975.)

Br. J. Pharmac. (1976), **56**, 235–239

HYPOTHALAMO-PITUITARY ADRENOCORTICAL FUNCTION IN THE RAT AFTER TREATMENT WITH BETAMETHASONE

JULIA C. BUCKINGHAM & J.R. HODGES
Department of Pharmacology, Royal Free Hospital School of Medicine, London

1 Hypothalamo-pituitary-adrenocortical (HPA) activity was suppressed in rats treated with betamethasone.

2 Recovery of normal HPA function occurred after corticosteroid withdrawal.

3 Although corticotrophin release was rapidly restored to its basal rate there was a delay in the return of the normal adrenocorticotrophic response to stress and normal adrenocortical function was evident only after the plasma adrenocorticotrophic hormone had reached 'supra-normal' levels.

4 The physiological significance and possible clinical relevance of the results are discussed.

Introduction

Corticosteroid treatment results in impairment of hypothalamo-pituitary adrenocortical (HPA) activity which appears to be due both to failure of the pituitary gland to mobilize adrenocorticotrophic hormone (ACTH) and to inability of the adrenal cortex to respond to the hormone. The HPA system recovers from corticosteroid-induced suppression in a way which is still not understood despite the existence of data on this aspect of HPA function both in man (Daly, Myles, Bacon, Beardwell & Savage, 1967) and in the rat (Hodges & Mitchley, 1970a). Such studies are often limited by the fact that only indirect indices of pituitary adrenocorticotrophic activity have been used. This paper describes the results of experiments in the rat in which direct estimates of circulating ACTH were made after betamethasone treatment.

Methods

Animals

Male albino Sprague-Dawley rats, obtained from a closed colony (Fisons Pharmaceuticals Ltd.), were kept at a constant temperature of 22°C in stock cages in a room where the experiments were performed. Food and water were available *ad lib.* Animals, weighing 100–125 g, were housed two per cage 3 days before the beginning of the experiments and handled three times a week from then onwards (Hodges & Mitchley, 1970b).

Betamethasone

Betamethasone (Betnelan, Glaxo) was administered to rats in the drinking water in concentrations of 20.0, 2.0 and 0.5 μg/ml for periods of 24 h, 13 days and 7 weeks respectively. These dose schemes were such that all animals ingested a total dose of approximately 450 μg betamethasone/100 g body weight (Hodges & Mitchley, 1970a, c). At the end of the treatment period the steroid solution was withdrawn abruptly and replaced with tap water. At various times after the cessation of treatment adrenal weights, plasma corticosterone concentrations, and plasma ACTH concentrations before and after stress were measured to assess the functional activity of the HPA system.

Collection of blood

Animals were killed by rapid decapitation. Blood was collected from the trunks into chilled heparinized tubes. The plasma was divided into two portions and stored at −30°C for ACTH estimation (Chayen, Loveridge & Daly, 1972, modified by Alaghband-Zadeh, Daly, Bitensky & Chayen, 1974) and at −4°C for corticosterone estimation (Zenker & Bernstein, 1958).

Stress

Rats were exposed for 1 min to ether vapour in a concentration sufficient to induce anaesthesia. Blood samples for ACTH estimation, were collected 2.5 min after the onset of anaesthesia (Hodges & Vernikos, 1960).

Removal of adrenal glands

Adrenal glands were removed from treated and untreated control animals immediately after the

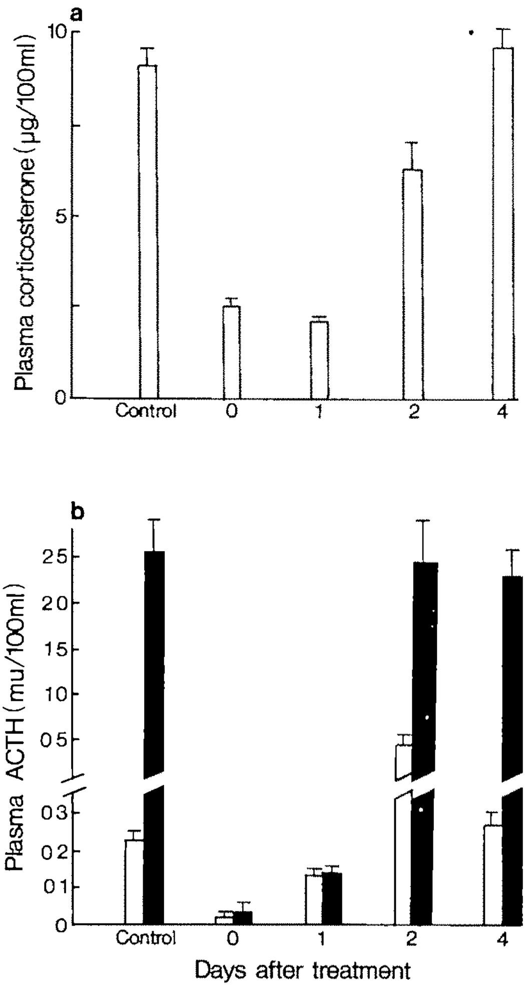


Figure 1 (a) Plasma corticosterone and (b) plasma ACTH concentrations in rats after 24 h treatment with betamethasone (20 µg/ml) in drinking water. Open columns, before ether stress; closed columns, 2.5 min after ether stress Each column shows mean of six observations Vertical lines indicate s.e. mean.

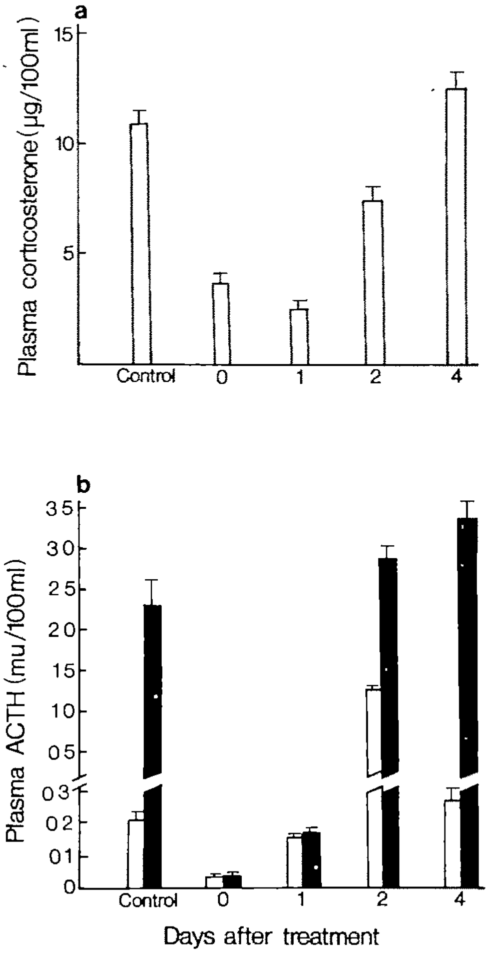


Figure 2 (a) Plasma corticosterone and (b) plasma ACTH concentrations in rats after 13 days treatment with betamethasone (2 µg/ml) in drinking water The results are expressed as in Figure 1.

collection of blood. Each gland was dissected free from periadrenal tissue and weighed on a torsion balance.

Results

Overnight treatment

The results obtained in rats which received betamethasone in their drinking water (20 µg/ml) for 24 h are shown in Figure 1. Overnight treatment with this high concentration of betamethasone resulted in suppression of HPA activity although there was no reduction in adrenal size (18.6 ± 1.2 mg/100 g body weight compared with untreated controls, 19.2 ± 1.4 mg/100 g body weight). The plasma corticosterone concentration was reduced but two days after steroid withdrawal it had risen significantly ($P<0.001$) to 6.2 ± 0.8 µg/100 ml, reaching the control value (9.0 ± 0.54 µg/100 ml) within 4 days. The plasma ACTH concentration in the same animals was also reduced considerably by the treatment to 0.029 ± 0.001 mu/100 ml. Twenty-four hours after withdrawal of the steroid it had risen significantly (0.137 ± 0.015 mu/100 ml) but was still lower than the control value (0.23 ± 0.03 mu/100 ml). It continued to rise and within 2 days reached a maximum value of 0.50 ± 0.07 mu/100 ml which was significantly ($P < 0.01$) higher than normal. It then declined. Stress caused a rise in plasma ACTH concentration in untreated controls from 0.23 to 2.55 mu/100 ml. The stress-induced release of the hormone was completely inhibited immediately and 24 h after the treatment. However within 2 days the response to stress had returned.

Thirteen day treatment

The results obtained in rats which received betamethasone in their drinking water (2 µg/ml) for thirteen days are shown in Figure 2. This treatment resulted in severe suppression of HPA activity with marked adrenal atrophy (11.3 ± 1.6 mg/100 g compared with 16.6 ± 1.4 mg/100 g in control rats). The adrenal weight returned to normal within 4 days of cessation of treatment (Table 1). The plasma corticosterone concentration was significantly ($P < 0.001$) reduced from 10.92 ± 0.69 µg/100 ml to 3.85 ± 0.49 µg/100 ml by the treatment. It rose to 7.58 ± 0.62 µg/100 ml within 2 days of withdrawing the steroid and on the 4th day it was not significantly ($P > 0.05$) different from the control value. The plasma ACTH concentration was markedly reduced (0.039 ± 0.001 mu/100 ml compared with 0.21 ± 0.031 mu/100 ml in the untreated group). However, two days after stopping the treatment it rose to 1.26 ± 0.12 mu/100 ml which was significantly ($P < 0.001$) higher than in the controls. The plasma ACTH concentration then declined returning, within 4 days, to the control value. The rise in plasma ACTH concentration which normally occurs in response to stress was absent immediately and 24 h after withdrawing the betamethasone. However, some response was evident on the second day and it was normal on the fourth day after withdrawal of the steroid.

Table 1 Adrenal size in rats after 13 days treatment with betamethasone in drinking water (2 µg/ml)

Days after withdrawal of betamethasone	*Adrenal wt (mg/100 g body wt ± s.e. mean)*
0	11.3 ± 1.6*
1	10.2 ± 1.5*
2	14.3 ± 1.7
4	15.6 ± 1.9
Controls	16.6 ± 1.4

Values are mean of six determinations ± s.e. mean.
* Significantly different from the control value ($P < 0.01$).

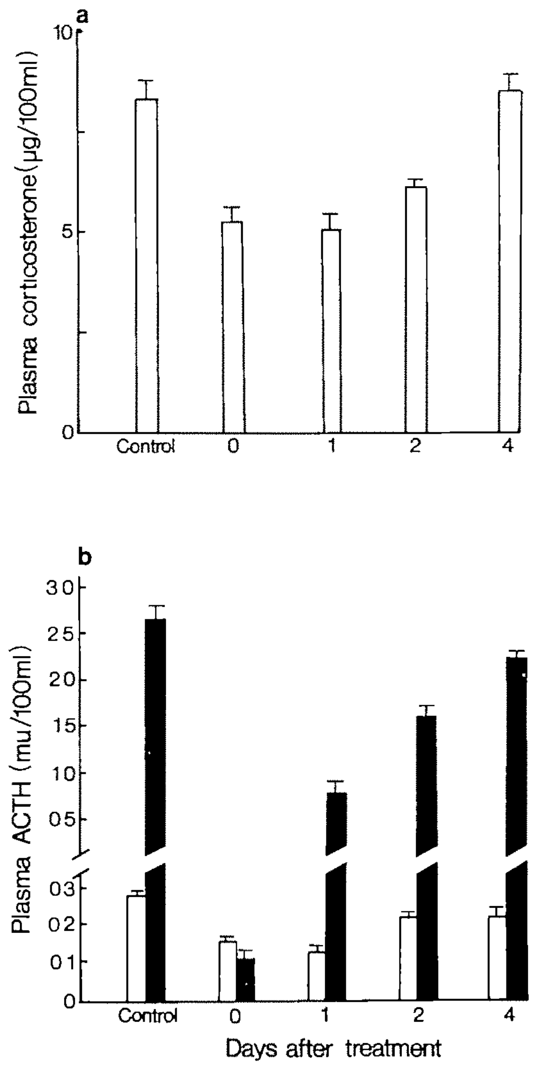


Figure 3 (a) Plasma corticosterone and (b) plasma ACTH concentrations in rats after 7 weeks treatment with betamethasone (0.5 µg/ml) in drinking water The results are expressed as in Figure 1.

Seven weeks treatment

The results obtained in rats which received betamethasone in their drinking water (0.5 µg/ml) for seven weeks are shown in Figure 3. This treatment with the lowest concentration of betamethasone resulted in only mild suppression of HPA activity. There was no adrenal atrophy when the treatment was withdrawn (10.62 ± 0.5 mg/100 g compared with 11.2 ± 0.6 mg/100 g in the untreated group). The plasma corticosterone concentration was significantly ($P<0.01$) reduced but only to about 60% of the control value (5.27 ± 0.38 µg/100 ml compared with 8.33 ± 0.43 µg/100 ml). After 2 days it had risen to 6.10 ± 0.20 µg/100 ml and was normal within 4 days. The plasma ACTH concentration was also found to be reduced by about 60% immediately and 24 h after the end of the treatment. However it rapidly returned to normal and was not significantly ($P<0.01$) different from the control value (0.29 ± 0.01 mu/100 ml) within 2 days. No rise in plasma ACTH occurred in response to stress immediately after the treatment. Twenty-four hours later there was a small but significant ($P<0.01$) stress-induced rise from 0.14 to 0.76 mu ACTH/100 ml. Within 4 days the response was normal.

Discussion

The low plasma ACTH and corticosterone concentrations reflect the marked suppression of HPA activity which betamethasone treatment produces. This HPA inhibition has been shown to be due to failure of the pituitary to mobilize endogenous ACTH and impairment of adrenocortical sensitivity to the hormone (Graber, Ney, Nicholson, Island & Liddle, 1965; Hodges & Sadow, 1969; Hodges & Mitchley, 1970a). However most of the workers in this field have used only either indirect or insensitive methods for the assessment of pituitary adrenocorticotrophic activity which do not show clearly how the HPA system recovers from such inhibition. The results described in this paper indicate that soon after the steroid has been withdrawn the pituitary gland begins to secrete ACTH and the adrenal size is rapidly restored to normal. The 'basal' plasma ACTH concentration rises quickly to a 'supra-normal' level but there is no concomitant rise in plasma corticosterone concentration indicating that the adrenal sensitivity must still be impaired. This suggests that an elevated 'basal' level of blood ACTH is necessary to 'prime' the adrenal cortex and thus restore adrenal sensitivity to normal (Graber *et al.*, 1965). The mechanism leading to the hypersecretion of ACTH is not known but it may well be associated with negative feedback regulation of basal ACTH secretion (Buckingham & Hodges, 1974, 1975).

Acute oral administration of high doses of corticosteroids to guinea-pigs and rats has been reported to leave adrenal sensitivity to ACTH unaffected immediately after the treatment (Purves & Sirett, 1965; Hodges & Mitchley, 1970c; Hodges & Hotston, 1971). However, the present work suggests that there is a reduction in adrenal activity since 48 h after overnight treatment with betamethasone, despite the hypersecretion of ACTH which then occurs, there is no concomitant rise in plasma corticosterone concentration. Adrenocortical insensitivity to ACTH is believed to be the direct result of abnormally low circulating concentrations of the hormone (Holub, Jailer, Kitay & Frantz, 1959; Daly *et al.*, 1967) and hence the delay in the development of adrenal insensitivity may be explained by the persistence for at least 24 h of an abnormally low blood ACTH concentration.

Although the capacity of the pituitary gland to maintain 'non-stress' plasma levels of ACTH returned rapidly after the withdrawal of betamethasone, there was a marked delay before it exhibited the normal adrenocorticotrophic response to a stressful stimulus. This dissociation confirms earlier studies which suggest that the mechanisms controlling the release of ACTH under 'basal' and 'stressful' conditions are different (Zimmerman & Critchlow, 1965, 1969; Hodges & Mitchley, 1970a, c; Buckingham & Hodges, 1974).

The present work indicates that the degree of HPA suppression is not related to the total amount of steroid administered. Our rats were given the same total dose of steroid over different periods and different effects on the HPA axis were evident. This confirms earlier studies (Hicklin & Wills, 1968; Hodges & Mitchley, 1970a) but is contrary to the findings of Treadwell, Savage, Sever & Copeman (1963). Prolonged treatment with the lower concentration of the steroid caused only slight suppression of HPA activity. It has been suggested, on the basis of observations in man, that steroids may have to reach a critical level in the blood and tissues before inhibition of HPA function develops (Shuster & Williams, 1961; Daly *et al.*, 1967; Knol, 1969) and that minimal HPA disturbance may follow prolonged treatment with very small doses of the steroid when there is no cumulative effect. Our results are in accord with such a hypothesis and add further support to the importance of properly designed and controlled studies on laboratory animals to assist in the solution of clinical problems associated with corticosteroid therapy.

We are grateful to the Medical Research Council and the Wellcome Trust for Grants for equipment and technical assistance, materials and animals respectively. We are also grateful to Miss Jacqueline Wale for technical assistance, to the World Health Organization for Third Corticotrophin I.W.S. and to Glaxo Laboratories Ltd. for betamethasone.

References

ALAGHBAND-ZADEH, J., DALY, J.R., BITENSKY, L. & CHAYEN, J. (1974). The cytochemical section assay for corticotrophin. *Clinical Endocrinology*, **3**, 319–327.

BUCKINGHAM, J.C. & HODGES, J.R. (1974). Interrelationships of pituitary and plasma corticotrophin with plasma corticosterone in adrenalectomized and stressed adrenalectomized rats. *J. Endocr.*, **63**, 213–222.

BUCKINGHAM, J.C. & HODGES, J.R. (1975). Interrelationships of pituitary and plasma corticotrophin and plasma corticosterone during adrenocortical regeneration in the rat. *J. Endocr.*, **67**, 411–417.

CHAYEN, J., LOVERIDGE, N. & DALY, J.R. (1972). A sensitive bioassay for adrenocorticotrophic hormone in human plasma. *Clinical Endocrinology*, **1**, 219–233.

DALY, J.R., MYLES, A.B., BACON, P.A., BEARDWELL, C.G. & SAVAGE, O. (1967). Pituitary and adrenal function during corticosteroid withdrawal in rheumatoid arthritis. *Ann. rheum. Dis.*, **26**, 18–25.

GRABER, A.L., NEY, R.L., NICHOLSON, W E., ISLAND, D.P. & LIDDLE, G.W. (1965). Natural history of pituitary-adrenal recovery following long-term suppression with corticosteroids. *J. clin. Endocr. Metab.*, **25**, 11–16.

HICKLIN, J A. & WILLS, M R (1968). Plasma 'cortisol' response to synacthen in patients on long-term small dose prednisolone therapy. *Ann. rheum. Dis.*, **27**, 33–37.

HODGES, J R. & HOTSTON, R.T. (1971). Suppression of adrenocorticotrophic activity in the ascorbic acid deficient guinea-pig. *Br. J. Pharmac.*, **42**, 595–602.

HODGES, J.R & MITCHLEY, S. (1970a). Recovery of hypothalamo-pituitary-adrenal function after prolonged treatment with betamethasone. *Br. J. Pharmac.*, **40**, 732–739.

HODGES, J.R. & MITCHLEY, S. (1970b). The effect of 'training' on the release of corticotrophin in response to minor stressful procedures in the rat. *J. Endocr.*, **47**, 253–254.

HODGES, J.R & MITCHLEY, S (1970c). The effect of betamethasone on circadian and stress-induced pituitary-adrenocortical function in the rat. *Br. J. Pharmac.*, **38**, 719–724.

HODGES, J.R & SADOW, J. (1969). Hypothalamo-pituitary-adrenal function in the rat after prolonged treatment with cortisol. *Br. J. Pharmac.*, **36**, 489–495.

HODGES, J.R. & VERNIKOS, J. (1960). The effect of hydrocortisone on the level of corticotrophin in the blood and pituitary glands of adrenalectomized and stressed adrenalectomized rats. *J. Physiol., Lond.*, **150**, 683–693.

HOLUB, D.A., JAILER, J W., KITAY, J.I. & FRANTZ, A.G. (1959). Direct and indirect estimation of pituitary adrenocorticotrophin reserves in man following adrenal steroid therapy. *J. clin. Endocr. Metab.*, **19**, 1540–1522.

KNOL, K. (1969). The function of the adrenal glands during prolonged corticosteroid therapy. *Scand. J. Resp. Dis. Suppl.*, **68**, 41–48.

PURVES, H D & SIRETT, N.E. (1965). Assay of corticotrophin in dexamethasone treated rats. *Endocrinology*, **77**, 366–374.

SHUSTER, S. & WILLIAMS, I.A. (1961). Pituitary-adrenal function during administration of small doses of corticosteroids. *Lancet*, **ii**, 674–678.

TREADWELL, B.L.J., SAVAGE, O., SEVER, E.D. & COPEMAN, W.S C. (1963). Pituitary-adrenal function during corticosteroid therapy. *Lancet*, **i**, 355–358.

ZENKER, N. & BERNSTEIN, D E (1958). The estimation of small amounts of corticosterone in rat plasma. *J. biol. Chem.*, **231**, 695–701.

ZIMMERMAN, E. & CRITCHLOW, V. (1965). Dissociation of feedback control of ACTH release. Compound 'B' in corticoid treated rats before and after stress. *Fed. Proc.*, **24**, 191.

ZIMMERMAN, E. & CRITCHLOW, V. (1969). Effects of intra-cerebral dexamethasone on pituitary-adrenal function in female rats. *Amer. J. Physiol.*, **217**, 392–396.

(Received October 28, 1975.)

INSTRUCTIONS TO AUTHORS

Papers will be considered for publication on all aspects of drug action, including chemotherapy. A recent issue of the Journal is a good guide to style.

Manuscripts (two copies) should be sent to Dr H.M Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh, EH8 9JZ, from whom copies of the full Instructions to Authors, published in *Br. J. Pharmac,* **50**, 3-23, may also be obtained. Specific points to note are·

1. **Manuscript** should be typed in double spacing on one side of paper not larger than A4 (206 x 294 mm). There should be a separate title page giving the names and addresses of the authors **in alphabetical order.** A short title of not more than 50 letters should also be suggested.

2. **Statement** Papers are accepted only if accompanied by a statement that they have not been and will not be published in whole or in part in any other Journal.

3. **Summary** (2 copies) This should be a short summary of results and conclusions arranged in numbered paragraphs.

4. **Abstract** (2 copies) An abstract of about 100 words suitable for inclusion in an abstracting journal should be typed on a separate sheet, giving the author's name and address and the title of the paper.

5. **Figures** These should not be larger than A4 (206 x 294 mm) and may be in the form of original drawings, recorded tracings or high quality photographic prints made from them. Negative prints of kymograph tracings (black on white) should be provided, and both ordinates and abscissae should be calibrated. Lettering on figures should be inserted in pencil. Symbols which are to appear in figures or legends should be chosen from the following:

○ ● □ ■ △ ▲ ▽ ▼

Photographs and photomicrographs should be printed on glossy paper and should be larger than the size required for reproduction.

6. **Tables** These should not have more than 85 characters to a line (counting spaces between columns as 4 characters) and certainly not more than 110 characters to a line, unless absolutely unavoidable. **Figure legends** and **tables** should be typed on separate sheets of paper.

7. **References** These should be collected in alphabetical order at the end of the paper. They should include the authors' names, year of publication (in brackets), title of article, title of publication (abbreviated in accordance with the fourth edition of the *World List of Scientific Periodicals*), volume number and first and last page numbers. References to books should in addition, include the names of editors, the edition number, where appropriate, and the town of origin and name of publisher.

Two page proofs will be supplied, one of which may be retained by the authors. The other should be corrected immediately and returned to the Press Editor. Corrections should be kept to a minimum.

Twenty-five reprints will be supplied to the authors free of charge. Additional reprints can be purchased. A reprint order form, which should be returned promptly, will be sent out with the proofs. No reprints of abstracts are supplied.

Short Communications These may be accepted if they merit priority publication and do not require revision. They may contain up to 1,200 words of text and only one figure or table. The summary should be a single paragraph. An abstract and statement should be included.

British Journal of Pharmacology

VOLUME 56 NUMBER 2 FEBRUARY 1976

133 MARLEY, E. & WHELAN, JENNIFER E. Some pharmacological effects of *p*-chlorophenylalanine unrelated to tryptophan hydroxylase inhibition.

145 BUTTAR, H.S. Impairment in the hepatic clearance of $[^{35}S]$-bromosulphophthalein in paracetamol-intoxicated rats.

155 BRANDTZAEG, P., GAUTVIK, K.M., NUSTAD, K. & PIERCE, J.V. Rat submandibular gland kallikreins: purification and cellular localization.

169 JURKIEWICZ, A. & JURKIEWICZ, N.H. Dual effect of α-adrenoceptor antagonists in rat isolated vas deferens.

179 ALPS, B.J., DEVOY, P.W. & WATERFALL, J.F. The evaluation of the novel pressor activity of γ-piperidinobutyramide (Wy 20051, DF480).

187 SAXENA, P.N. Sodium and calcium ions in the control of temperature set-point in the pigeon.

193 EVANS, JANE P.M., GRAHAME-SMITH, D.G., GREEN, A.R. & TORDOFF, ANN F.C. Electroconvulsive shock increases the behavioural responses of rats to brain 5-hydroxytryptamine accumulation and central nervous system stimulant drugs.

201 SEIDLER, F.J. & SLOTKIN, T.A. Effects of chronic nicotine administration on the denervated rat adrenal medulla.

209 MICHAL, F. & MOTAMED, MINA. Shape change and aggregation of blood platelets: interaction between the effects of adenosine diphosphate, 5-hydroxytryptamine and adrenaline.

219 ABAITEY, A.K. & PARRATT, J.R. Cardiovascular effects of diethylcarbamazine citrate.

229 FLOWER, R.J., HARVEY, E.A. & KINGSTON, W.P. Inflammatory effects of prostaglandin D_2 in rat and human skin.

235 BUCKINGHAM, JULIA C. & HODGES, J.R. Hypothalamo-pituitary adrenocortical function in the rat after treatment with betamethasone.

Printed in Great Britain by The Whitefriars Press Ltd., London and Tonbridge

British Journal of Pharmacology

March 1976

BRITISH COUNCIL [illegible]

21/5

NOT TO [illegible]

Edited for the British Pharmacological Society by

A.W. Cuthbert *(Chairman)* H.M Adam *(Secretary)*
Margaret Day *(Press Editor)*
J.D. Stephenson *(Assistant Press Editor)*

A.M. Barrett
W G. Bisset
T.B. Bolton
D.A. Brown
B A. Callingham
T.J. Crow
F. de Matteis
C.T. Dollery
V Eisen
E.W. Gill
A.F. Green
D.F Hawkins
Margarethe Holzbauer
D.B. Hope
J.S. Kelly
G.M. Lees
G.P. Lewis
I.H.M Main
E.K. Matthews
J.F. Mitchell
P.A. Nasmyth
M.W. Parkes
M. Sandler
A.P. Silverman
M.A Simmonds
F.M. Sullivan
A Ungar
R A. Webster

Papers will be considered for publication on all aspects of drug action, including chemotherapy.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh EH8 9JZ. Authors should consult the instruction inside the back cover.

The British Journal of Pharmacology is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633. ISSN 0007-1188.

Annual subscription £36.00 ($90 USA and Canada). (Orders must be accompanied by remittance. Prices applicable only to orders received before 31st December 1976.)

Cheques should be made payable to Macmillan Journals Limited: Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

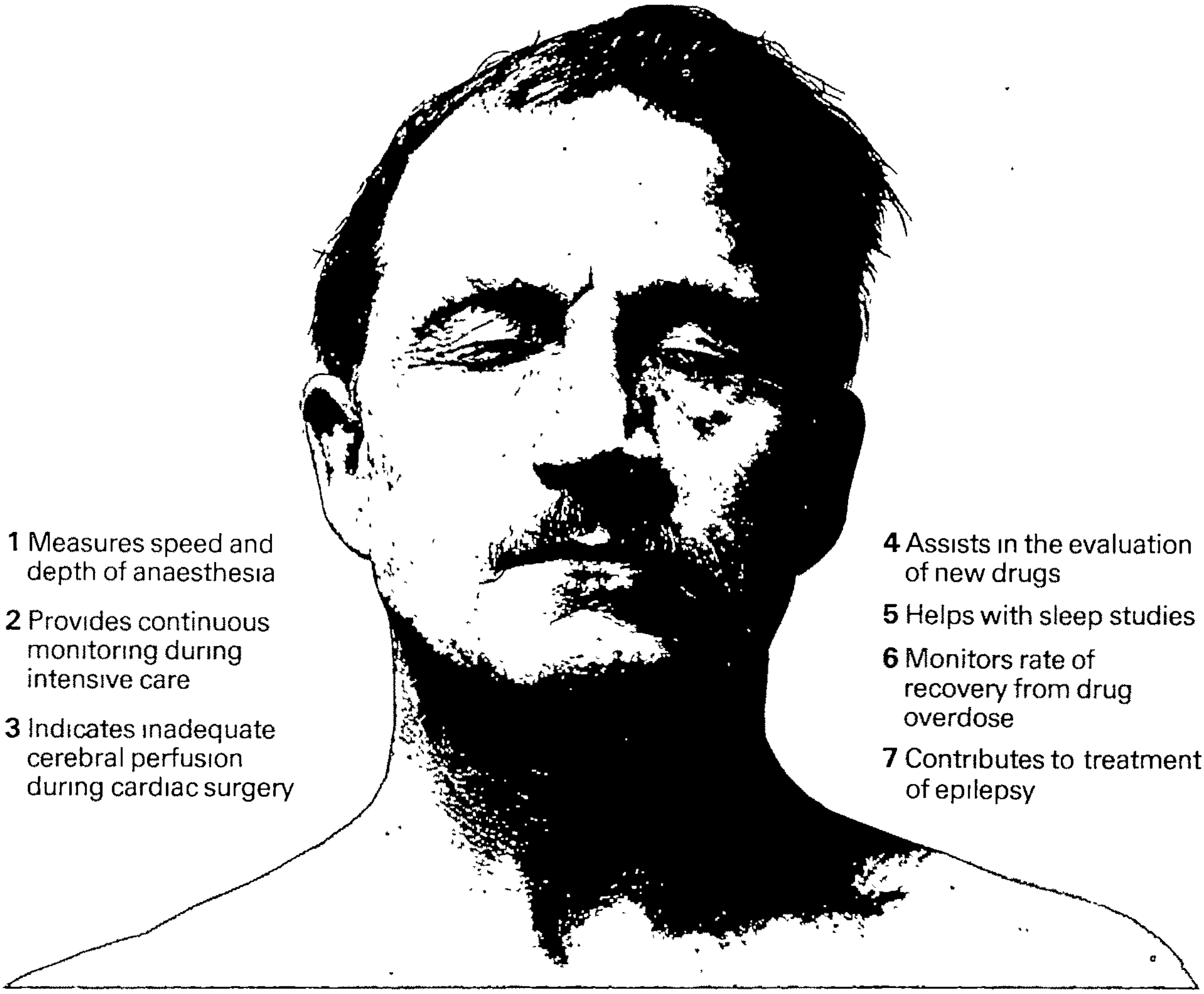
Enquiries concerning advertising space and rates should be addressed to. Advertisement Department, Macmillan Journals Limited, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633.

Copyright © 1976 by the *British Journal of Pharmacology.* All rights of reproduction are reserved in respect of all papers, articles, illustrations, etc., published in this journal in all countries of the world.

Second class postage paid at New York, N.Y
US Mailing Agent, Air and Sea Freight Inc., 527 Madison Avenue, New York, NY 10022.

Devices

Continuous Cerebral Function Monitor.



1 Measures speed and depth of anaesthesia

2 Provides continuous monitoring during intensive care

3 Indicates inadequate cerebral perfusion during cardiac surgery

4 Assists in the evaluation of new drugs

5 Helps with sleep studies

6 Monitors rate of recovery from drug overdose

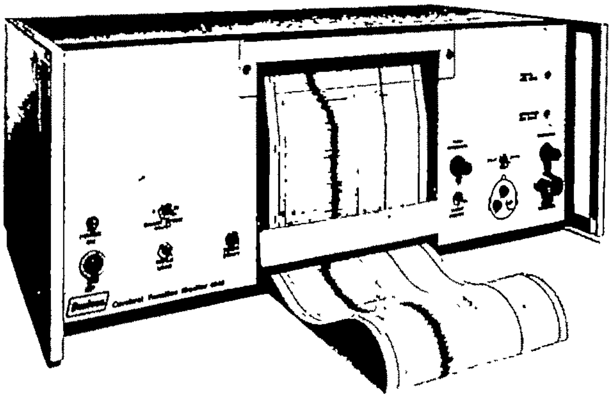
7 Contributes to treatment of epilepsy

The compact and economic two-channel Cerebral Function Monitor from Devices provides continuous and reliable monitoring of cerebral activity for a broad range of vital surgical, intensive care and research applications

It complements conventional EEG recordings by providing information over long periods of time, without the need for constant attention from skilled personnel. In intensive care, nursing staff can operate and interpret the instrument with ease.

Compared to 10,000 cm of chart paper per hour for standard EEG recordings, the CFM uses only 6 cm/hr for intensive therapy and 30 cm/hr for theatre recordings.

Experience that shows.



For full specification details and
examples of CFM waveforms just
write or telephone.

Devices

Devices limited, Instrument Division,
Hyde Way, Welwyn Garden City,
Hertfordshire, AL7 3AP.
Telephone: Welwyn Garden 28511.

British
Journal of
Clinical
Pharmacology

The British Journal of Clinical Pharmacology is the authoritative channel of communication between clinical and academic research and that of the pharmaceutical industry. Published bi-monthly, the journal aims to encourage the cross-fertilisation of information and ideas, to help avoid the dangers of compartmentalisation.

BJCP contains original papers and reports on all aspects of drug action in man. These include the pre-clinical testing of drugs in normal volunteers and important clinical trials in patients, using the latest techniques.

Editorial provides informed comment and evaluation of papers in terms of contemporary clinical practice, while Letters to the editors allow immediate discussion of urgent observations and negative reports.

Recent papers include:
Physiological and psychological effects of ±-propranolol, +-propranolol and diazepam in induced anxiety, P. J. Tyrer & M. H. Lader.
Acute effect of antipyretic analgesics, alone or in combination with alchohol, on human psychomotor skills related to driving, M. Linnoila, T. Seppala & M. J. Mattila.
Effect of phenobarbitone on hepatic drug-metabolizing enzymes and urinary d-glucaric acid excretion in man, D. S. Lecamwasam, C. Franklin & P. Turner.

Annual subscription £24.00 (USA and Canada $55.00) Orders must be accompanied by remittance. Prices applicable only to orders started before 31st December 1976. Cheques should be made payable to: Macmillan Journals Ltd., Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

Pitman Medical

pm

ADVANCED MEDICINE

Topics in Therapeutics—1

Edited by A.M. BRECKENRIDGE, *MD, MSc, FRCP*

The first volume in a new series of Symposia held by the Royal College of Physicians of London, being an extension of, and complementary to, the well-established Advanced Medicine symposia. The new series concentrates entirely on therapeutics, while the established series will continue to cover the wide range of the whole of modern medicine.

Contents: Problems of Poisoning and Drug Overdosage—New Problems Presented by Old Drugs—Medical Education and the Drug Explosion—Problems of Changing Therapeutic Practice—Problems Presented by Psychotropic Agents.

270 pages Illustrated Paperback £6.50 net

FITC-DEXTRANS

for permeability and microcirculation studies in animals

Well characterized tracer substances

- fluorescein label for easy detection
- ultra-micro determination in nanolitre sample volumes
- narrow molecular weight distribution
- five fractions between 3,000 and 150,000 daltons

FITC-Dextrans and other products for biological research are described in the booklet "Biomedical Research. Product guide", free on request.

Pharmacia (Great Britain) Ltd.
Paramount House,
75 Uxbridge Road,
London W5 5SS

Pharmacia
Fine Chemicals

JUST PUBLISHED

TOXICOLOGY

By L. J. CASARETT PhD,
late Professor of Pharmacology at the University of Hawaii School of Medicine, Honolulu

and J. DOULL M D, PhD,
Professor of Pharmacology and Toxicology, University of Kansas School of Medicine, Kansas City with 29 Contributors

MAIN UNITS: –

1) General Principles of Toxicology.
2) Systemic Toxicology
3) Toxic Agents
4) Applications of Toxicology

This important new book will be of interest not only to toxicologists and pharmacologists, but also to those concerned with environmental problems, agricultural and food technology.

1975 • 768 pp 15 illus. • £14.50

Price is subject to change without notice

BAILLIÈRE
TINDALL
7/8 Henrietta Street
London WC2E 8QE

British Journal of Pharmacology

INDEX

(1966–1973)

The eight year cumulative index (1966–1973) to the British Journal of Pharmacology is now available.

Price £10.45 (including postage)

Available from:
DEPARTMENT AS11,
MACMILLAN JOURNALS LTD,
BRUNEL ROAD,
BASINGSTOKE, RG21 2XS,
HAMPSHIRE, ENGLAND.

Orders must be accompanied by remittance Cheques should be made payable to Macmillan Journals Ltd Payment may be made in any currency at the current exchange rate

---

DRUGS AND CENTRAL SYNAPTIC TRANSMISSION

Edited by P. B. Bradley and B. N. Dhawan

Macmillan Pharmacology Monographs

Even though drugs have been used as research tools in pharmacological, physiological and neurochemical studies for a long time, the symposium reported in this book was perhaps the first interdisciplinary meeting where this particular use of drugs was the main theme. Disciplines represented include electrophysiology, neurochemistry, biochemistry and behavioural sciences.

about £12.50 May 1976

For further details please write to Anne Calcott, The Macmillan Press, Little Essex Street, London WC2R 3LF.

M MACMILLAN PRESS

VOLUME 56(3) MARCH 1976

British Journal of Pharmacology

245 KOSS, M.C. & NAKANO, J. Reflex bradycardia and hypotension produced by prostaglandin $F_{2\alpha}$ in the cat

255 ASHFORD, J. & JONES, B.J. The effects of intra-amygdaloid injections of 6-hydroxydopamine on avoidance responding in rats

263 LEES, P. & SERRANO, L. Effects of azaperone on cardiovascular and respiratory functions in the horse

271 CLARKE, D.E. ROMANYSHYN, W.M. Blood pressure responses to adrenal field stimulation as a measure of adrenal catecholamine release

279 DORRIS, R.L. & SHORE, P.A. On the mechanism of action of clozapine on the adrenergic neurone

285 GUTMAN, Y. & MAZUR-RUDER, M. Rat uterus renin-like activity: effect of stimuli and hormones

293 HIMORI, N. & TAIRA, N. A method for recording smooth muscle and vascular responses of the blood-perfused dog trachea *in situ*

301 RIVERA-CALIMLIM LEONOR. Impaired absorption of chlorpromazine in rats given trihexyphenidyl

307 CURTIS, D.R., GAME, C.J.A. & LODGE, D. Benzodiazepines and central glycine receptors

313 DICK, W.C., GRENNAN, D.M. & ZEITLIN, I.J. Studies on the relative effects of prostaglandins, bradykinin, 5-hydroxytryptamine and histamine on the synovial microcirculation in dogs

317 CHAHL, LORIS A. & LADD, R.J. The effects of prostaglandins E_1, E_2 and $F_{2\alpha}$ on the cutaneous vasculature of the rat

323 BOWERY, N.G. & JONES, G.P. A comparison of γ-aminobutyric acid and the semi-rigid analogues 4-aminotetrolic acid, 4-aminocrotonic acid and imidazole-4-acetic acid on the isolated superior cervical ganglion of the rat

331 ANDERSON, A.A. & LEES, G.M. Investigation of occurrence of tolerance to bronchodilator drugs in chronically pretreated guinea-pigs

339P PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY, London, 17–19 December, 1975

PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY

Royal College of Surgeons, 17th–19th December, 1975

339P JONES, R.L. Selectivity of action of some C15-modified prostaglandins D

340P WHITTLE, B.J.R. Prostaglandins and changes in the gastric mucosal barrier and blood flow during indomethacin- and bile salt-induced mucosal damage

341P WILLIAMS, T.J. (introduced by Lewis, G.P.). The pro-inflammatory activity of E-, A-, D- and F-type prostaglandins and enalogues 16, 16-dimethyl-PGE_2 and (15S)-15-methyl-PGE_2 in rabbit skin; the relationship between potentiation of plasma exudation and local blood flow changes

342P CHANG, J., LEWIS, G.P. & PIPER, PRISCILLA J. The effects of anti-inflammatory steroids on levels of prostaglandin in adipose tissue *in vitro*

343P BLACKWELL, G.J. & FLOWER, R.J. Effects of steroid hormones on tissue levels of prostaglandin 15-hydroxydehydrogenase in the rat

244P BUNTING, S., MONCADA, S., NEEDLEMAN, P. & VANE, J.R. Formation of prostaglandin endoperoxides and rabbit aorta contracting substance (RCS) by coupling two enzyme systems

345P FORD-HUTCHINSON, A.W., SMITH, M.J.H. & WALKER, J.R. Chemotactic activity of solutions of prostaglandin E_1

346P BAKER, DOROTHEA J, TRIST, D.G. & WEATHERALL, M. Inhibition of phagocytosis by mepacrine

347P ASSEM, E.S.K. Inhibition by antigen and by histamine antagonists of the uptake of histamine by isolated human leucocytes

349P BAKHLE, Y.S. The nature of the bradykinin inactivating system in isolated lungs

350P MORTON, I.K.M., ROSE, G.E., SAVERYMUTTU, S.H. & WOOD, J.R. Effects of some fatty acids on gallbladder function

351P AMBACHE, N. & KILLICK, S.W. Species variation in motor transmission to the retractor penis muscle

352P NISTRI, A. Effects of morphine on acetylcholine release from the frog spinal cord

353P GARTHWAITE, J. (introduced by Brown, D.A.). The uptake of weak acids and bases into isolated rat superior cervical ganglia in relation to intracellular pH

354P CHAPPLE, D.J., ENDERBY, D.H., HUGHES, R. & PAYNE, J.P. Neuromuscular and cardiovascular studies with dimethyl tubocurarine in anaesthetized cats, rhesus monkeys and man

355P HIROM, P.C., MILLBURN, P. & PARKER, R.J. (introduced by Williams, R.T.). The enterohepatic circulation of ^{1}H-phenolphthalein in the rat

356P RICHARDSON, B.P. (introduced by Berde, B.). The effects of cyproheptadine pretreatment on insulin release from isolated pancreatic islets

357P WAN, BEATRICE Y.C. (introduced by Black, J.W.). Effects of dibutyryl cyclic AMP and phosphodiesterase inhibitors on acid secretion by mouse stomach *in vitro*

358P RICHARDSON, P.D.I. The effects of intravenous secretin on the small intestinal vasculature of the cat

359P DUNCAN, A.J. & WILSON, W.S. Some preservatives in eyedrop preparations hasten the formation of dryspots in the rabbit cornea

360P HOLMES, S.W. & SUGDEN, D. The effect of melatonin on pinealectomy-induced hypertension in the rat

361P MELROSE, JOANNE, PALFREYMAN, M.G., POYSER, R.H. & WHITING, R.L. BRL 13776: a novel antihypertensive agent with interesting noradrenaline-depleting properties

362P BILSKI, A. & WALE, J. (introduced by Dawes, P.M.). Two types of intrinsic sympathomimetic activity with β-adrenoceptor blocking drugs

362P KOLLER, K. (introduced by Jenkinson, D.H.). An interaction between the α and β actions of catecholamines in guinea-pig liver slices

363P COLLIER, P.S. & SHANKS, R.G. Alterations to vasodilator effects of isoprenaline in the dog following intra-arterial infusions with isoprenaline, salbutamol and orciprenaline

364P BLAKELEY, A.G.H. & SUMMERS, R.J. The effects of AH 5158 on the overflow of transmitter and the uptake of $[^3H]$-(–)-noradrenaline in the cat spleen

365P DARGIE, H.J., FRANKLIN, S.S. & REID, J.L. The sympathetic nervous system and renovascular hypertension in the rat

366P FINCH, L. & HERSOM, A. Studies on the centrally mediated cardiovascular effects of apomorphine in the anaesthetized rat

366P BARASI, S., PARRY, O. & ROBERTS, M.H.T. The interpretation of responses of motoneurone field potentials to 5-hydroxytryptamine

367P GOODLET, I., MIREYLEES, S.E. & SUGRUE, M.F. The selective inhibition of 5-hydroxytryptamine re-uptake by Org 6582

368P CURZON, G. & MARSDEN, C.A. Effect of LSD on rat brain 5-hydroxytryptamine metabolism at elevated environmental temperature

370P LITTLETON, J.M. & MACLEAN, K.I. (introduced by Brownlee, G.). Alterations in dopamine uptake in rat corpus striatum induced by combinations of stress and Δ^8-tetrahydrocanabinol (Δ^8-THC)

371P JATON, A.L., LOEW, D.M. & VIGOURET, J.M. CF 25-397 (9, 10-Didehydro-6-methyl-8β-[2-pyridylthiomethyl]ergoline), a new central dopamine receptor antagonist

372P FRY, J.P., SHARMAN, D.F. & STEPHENS, D.B. The ontogenesis of cerebral dopamine metabolism in the pig

373P DONALDSON, I.M., JENNER, P., MARSDEN, C.D., MILLER, R. & PERINGER, E. Is metoclopramide a directly acting dopamine receptor antagonist?

374P CANDY, J.M. & KEY, B.J. Blockade of the electrocortical changes induced by perfusion of amphetamine in the brain stem reticular formation

375P YOUDIM, M.B.H. Monoamine oxidase active site; the binding to and titration of monoamine oxidase with $[^{14}C]$-selective inhibitors

375P HILLIDGE, C.J. & LEES, P. Influence of etorphine acepromazine and diprenorphine on cardiovascular function in ponies

376P GRANT, L. & POWIS, G. The effect of inhibitors of alcohol metabolism on the changes in the hepatic microsomal metabolism of foreign compounds produced by a single dose of ethanol in the rat

377P ABU-MURAD, C., GRIFFITHS, P.J. & LITTLETON, J.M. (introduced by Brownlee, G.). Catecholamine metabolism and liver dysfunction during induction of ethanol dependence

378P FILE, SANDRA E. (introduced by Neal, M.J.). Are central cholinergic pathways involved in the habituation of exploration and distraction?

379P AMADUCCI, L., MANTOVANI, P. & PEPEU, G. Effect of some phospholipids on acetylcholine output from the cerebral cortex in the rat

380P NAHORSKI, S.R., PATTON, WENDY & ROGERS, K.J. Developmental changes in the sensitivity of neurohormone-stimulated cyclic AMP formation in chick cerebral hemispheres

381P ANLEZARK, G.M., MARSDEN, C.D. & MELDRUM, B.S. A primate model of acute dystonic reaction to neuroleptics

382P COLLIER, H.O.J. & FRANCIS, D.L. Stereospecific suppression by opiates of the quasi-morphine abstinence syndrome elicited by 3-isobutyl-1-methylxanthine (IBMX)

382P HOLZBAUER, MARGARETHE. Physiological aspects of the hypnotic properties of steroid hormones.

383P ANLEZARK, G.M., HORTON, R.W., MELDRUM, B.S., SAWAYA, M.C.B. & STEPHENSON, J.D. γ-Aminobutyric acid metabolism and the anticonvulsant action of ethanolamine-o-sulphate and di-n-propylacetate

385P LEWIS, A.J. The assessment of systemically and topically administered anti-inflammatory drugs using u.v. erythema production in the rat

386P CUTHBERT, N.J., DINNEEN, L.C., FRANCIS, D.L. & SCHNEIDER, C. A rapid *in vivo* test for dependence potential of analgesic drugs

386P HAEUSLER, G. & LEWIS, P.J. Sympathetic nerve recording in the conscious rabbit

387P BRADLEY, N.J. & POULTER, L.W. (introduced by Lewis, G.P.). *In vitro* methods of assessing the physiological activation of macrophages *in vivo*

388P FRY, J.P., SHARMAN, D.F. & STEPHENS, D.B. The effect of apomorphine on oral behaviour in piglets

388P FARRINGTON, HELEN E. & OWEN, D.A.A. Measurement of vascular changes in acute inflammatory responses

389P BUSHMAN, J.A., ENDERBY, D.H., FOWLER, K.T. & PAYNE, J.P. The use of a mass spectrometer for the analysis and measurement of anaesthetic vapours

390P ENDERBY, D.H., HUGHES, R., PAYNE, J.P. & WORSLEY, R. Assessment of neuromuscular blockade in man by use of tetanic and single twitch contractions of the adductor pollicis muscle

391P HUTCHINGS, R.T., LEWIS, G.P., SABIKOWSKI, Z.T. & WESTWICK, J. Measurement of changes in the microvasculature

391P NIEDERHAUSER, U. & WHITTLE, B.J.R. A simple perfusion system for the study of histamine release from rat peritoneal mast cells

392P ADEBANJO, A.O., AMBACHE, N. & VERNEY, J. The inhibitory transmission to the internal anal sphincter

394P EDWARDS, J.H., EVANS ELIZABETH, EVANS, P.H., NICHOLLS, P.J. & RAJAN, K.T. Effect of a purified cotton dust extract on human lung in culture

395P DOLPHIN, A.C., ELLIOTT, P.N.C. & JENNER, P. Reduction of locomotor activity in mice by dopamine-β-hydroxylase inhibitors — evidence against the involvement of non-specific irritation

395P McGRATH, J.C. & MacKENZIE, J.E. A pithed rabbit preparation for stimulation of different segments of the autonomic outflow

396P BAKER, J.B.E. The episcope for large screen projection of tracings on opaque media during their recording for demonstrations

396P PECK, M.J. & WILLIAMS, T.J. (introduced by Lewis, G.P.). Measurement of inflammatory exudation and local blood flow changes in rabbit skin, using [^{131}I]-albumin and ^{133}Xe

396P LEWIS, G.P., PECK, M.J., WILLIAMS, T.J. & YOUNG, BEVERLEY A. Measurement of blood flow in rabbit skin homografts and autografts using a ^{133}Xe-clearance technique

396P McCALL ELAINE & YOULTEN, L.J.F. Prostaglandin production by rat polymorphonuclear leucocytes

Br. J. Pharmac. (1976), 56, 245–253

REFLEX BRADYCARDIA AND HYPOTENSION PRODUCED BY PROSTAGLANDIN $F_{2\alpha}$ IN THE CAT

M.C. KOSS & J. NAKANO
Department of Pharmacology, University of Oklahoma, College of Medicine,
Oklahoma City, Oklahoma 73190, U.S.A.

1 Intravenous administration of prostaglandin $F_{2\alpha}$ results in a dose-dependent increase in pulmonary arterial pressure, decrease in systemic arterial pressure and a delayed bradycardia. Pulmonary vasoconstriction was observed at doses as low as 0.1 and 0.3 µg/kg. The systemic depressor and heart rate lowering effects were observed at 1 µg/kg doses and above.

2 A moderate bradycardia was still observed after atropine blockade but was abolished following bilateral vagotomy. Neither of these procedures affected the pulmonary vascular response.

3 Injections of submaximal doses of prostaglandin $F_{2\alpha}$ (1–4 µg/kg) produced a greater and longer lasting bradycardia when injected into the left atrium than was observed following intravenous administration. In addition the latency of onset was much shorter following left atrial injection. These doses resulted in no change in heart rate and a minimal hypotension when injected into the brachiocephalic artery or into the aortic arch.

4 Small doses of prostaglandin $F_{2\alpha}$ administered at the level of the origin of the coronary arteries produced marked decreases in heart rate and blood pressure whereas no change occurred following injection of the same amount into the ascending aorta at more distal sites.

5 These results suggest that prostaglandin $F_{2\alpha}$ produces bradycardia and hypotension in the cat by activating 'receptors' located in the left heart or by acting on structures perfused by means of the coronary arteries.

Introduction

Prostaglandin $F_{2\alpha}$ is unique among the various prostaglandin sub-groups in that there are qualitative species variations with regard to its cardiovascular actions (Bergström, Carlson & Weeks, 1968; Nakano, 1973). In contrast to the uniformly depressant actions of the E and A prostaglandin subgroups, intravenous administration of prostaglandin $F_{2\alpha}$ results in a systemic pressor response in the dog and rat (Ducharme, Weeks & Montgomery, 1968) and a systemic depressor response in the cat and rabbit (Änggård & Bergström, 1963; Horton & Main, 1965). We have demonstrated that several haemodynamic parameters are responsible for the depressor action of prostaglandin $F_{2\alpha}$ in the cat (Koss & Nakano, 1973; Koss, Gray, Davison & Nakano, 1973a). These include: (1) direct, but transient, peripheral vasodilatation, (2) decreased cardiac output as a result of increased pulmonary arterial pressure and (3) marked delayed bradycardia.

The present study was undertaken to analyze further the prostaglandin $F_{2\alpha}$-induced bradycardia and hypotension in the cat. Following an analysis of the dose-response relationships, an attempt was made to determine what role afferent as well as efferent vagal mechanisms might play in this response. This was accomplished by observing haemodynamic changes induced by prostaglandin $F_{2\alpha}$ before and after atropine and before and after bilateral vagotomy. In addition, different injection sites were used to identify possible sites of action for this agent. Our results suggest that intravenous prostaglandin $F_{2\alpha}$ acts on structures perfused by means of the coronary arteries. A preliminary report of this study has been published (Koss, Rieger & Nakano, 1973b).

Methods

Thirty-seven adult cats of either sex were anaesthetized with chloralose (60–80 mg/kg) given intraperitoneally. A femoral artery and vein were cannulated in order to measure systemic arterial blood pressure and for the intravenous administration of drugs. Following cannulation of the trachea, positive pressure respiration was initiated with a Harvard respirator. The animals were placed on their right sides and the left hemithorax was opened, usually between the fourth and ninth ribs. A branch of the pulmonary artery supplying either the left cardiac or left apical lobe was cannulated for direct measurement of pulmonary arterial pressure. We were careful during these procedures not to damage the vagal fibres near the heart. Pulmonary and systemic arterial pressures were recorded continuously by means of Statham P23BC and P23Dd pressure transducers. Heart rate was measured with a Grass tachograph (7P4D) and all responses were recorded continuously on a Grass polygraph (7B).

In the first group of cats (6) an attempt was made to determine the effects of increasing doses of prostaglandin $F_{2\alpha}$ on the three haemodynamic parameters measured. A total of five injections was administered intravenously to each animal starting at the lowest dose (0.1 μg/kg) and progressing sequentially in approximately three-fold steps to the highest dose (9.0 μg/kg).

In the second series of experiments (10 cats), the effect of intravenous prostaglandin $F_{2\alpha}$ was observed before and after administration of atropine sulphate (1.5–2 mg/kg). At the onset of each experiment, the dose of prostaglandin $F_{2\alpha}$ producing the maximal change in all variables was established. This was within the range of 6–12 μg/kg. The effectiveness of the atropine blockade was established in each experiment by the absence of cardiovascular responses following intravenous acetylcholine chloride (5–10 μg).

In the third group of experiments (9 cats) the responses to maximally effective intravenous doses of prostaglandin $F_{2\alpha}$ (6–12 μg/kg) were observed before and after bilateral vagotomy. Both vagi were isolated at the cervical level prior to the control injections of prostaglandin $F_{2\alpha}$. At least two responses were obtained in each experiment before vagotomy and only those cats with reproducible responses were used.

The final series of experiments (12 cats) was designed to determine the principle site of action of prostaglandin $F_{2\alpha}$. Doses necessary to produce a moderate or submaximal change in heart rate and blood pressure were used (1–4 μg/kg). Intra-arterial injections were made by means of polyethylene catheters, the tips of which were placed at several sites. Injections were made into (1) the left atrium, (2) the brachiocephalic artery, (3) the ascending aorta at the origin of the coronary arteries, (4) the ascending aorta distal to the exit of the coronaries and (5) the aortic arch and descending aorta. The left atrial injections were made by means of a catheter placed in the left atrial appendage. The other sites were reached by means of a catheter in a femoral artery. In order to place the catheter at the exit of the coronary arteries it was first threaded into the left ventricle and then slowly withdrawn until the characteristic left ventricular pressure wave was either intermittent or absent. In the two cases where this was possible the location of this catheter was confirmed post-mortem. The positioning at the other sites was by direct palpation in the open chest animal.

Crystalline powder prostaglandin $F_{2\alpha}$ (tromethamine salt) used in these experiments was supplied by Dr J.E. Pike, Upjohn Company, Kalamazoo, Michigan. A stock solution (1 mg/ml) was prepared with distilled water and stored at −10°C. Fresh solutions (10 or 100 μg/ml) were prepared shortly before the experiments by diluting the stock solution with 0.9% w/v NaCl solution (saline). After the prostaglandin solution was placed in the cannula it was flushed in with 2 ml of saline for 2–3 seconds. As multiple injections were made, a recovery period of at least 15 min was used. The total number of injections generally varied between three and five in each experiment. As shown previously (Koss & Nakano, 1973; Koss, *et al.*, 1973a) there is no apparent tachyphylaxis at these dose levels when at least 15 min elapses between injections.

Results

Intravenous injections of prostaglandin $F_{2\alpha}$ caused dose-related changes in pulmonary arterial pressure, systemic arterial blood pressure and heart rate. As shown in Figure 1, doses as low as 0.1 and 0.3 μg/kg resulted in a constriction of the pulmonary vasculature while having little or no effect on systemic blood pressure and heart rate. Larger doses of prostaglandin $F_{2\alpha}$ (above 1.0 μg/kg) produced a fall in systemic arterial blood pressure and a delayed bradycardia. The onset of the systemic depressor action varied between 6–15 seconds. At 9 μg/kg the mean onset was 8.4 ± 1.7 s and the maximal effect was seen in 49.8 ± 6.0 s, $n=6$. In contrast, the onset of the bradycardia was more delayed with a mean onset for the 9 μg/kg dose occurring in 27.8 ± 6.0 s and reaching its peak effect in 66.4 ± 9.7 s in these same preparations. Table 1 summarizes the dose-related haemodynamic effects of prostaglandin $F_{2\alpha}$ in six experiments. Occasionally a transient hypertension lasting between 5–15 s was observed following the injection. It is not known whether this is a drug effect or merely a result of the bolus injection.

Effect of atropine blockade

The responses to intravenous prostaglandin $F_{2\alpha}$ (6–12 μg/kg) were observed before and after blockade of efferent vagal mechanisms with atropine sulphate (1.5–2 mg/kg). This dose range produced maximal changes in the three parameters measured. As shown in Figure 2c, the increase in pulmonary arterial pressure was not altered following atropine whereas the bradycardia and hypotension were reduced. Initial blood pressure and heart rate levels in all of the experiments were similar to those in Figure 2 (a and b). The prostaglandin $F_{2\alpha}$-induced decrease in mean systemic arterial pressure averaged 56 ± 6 mmHg before and 30 ± 4 mmHg after atropine blockade

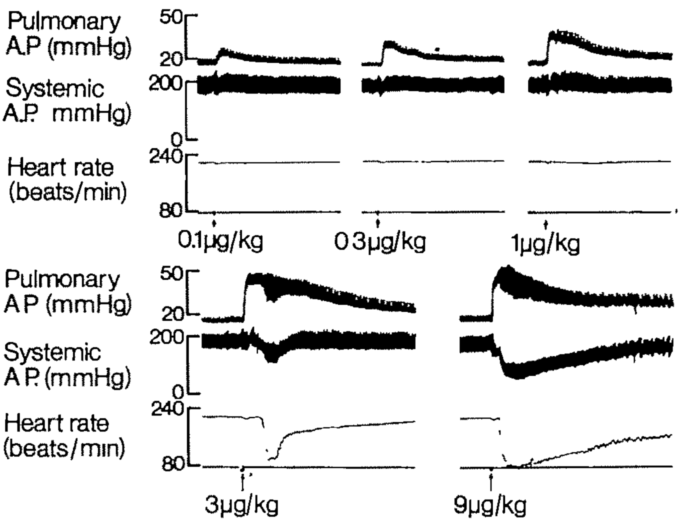


Figure 1 Effects of intravenous administration of prostaglandin F$_{2\alpha}$ (0.1 to 9 µg/kg) on pulmonary arterial pressure, systemic arterial pressure and heart rate in cat anaesthetized with chloralose. Doses were injected sequentially from the smallest to the largest at no less than 15 min intervals. Each time mark equals 5 seconds. Upper panels represent 4 min and lower panels represent 6 minutes

($P < 0.01$, $n = 10$). The decrease in heart rate averaged 48 ± 7 beats/min before and 10 ± 2 beats/min after atropine ($P < 0.01$, $n = 10$). A residual bradycardia and prolonged depressor response was observed in all of the atropine-treated preparations.

Effect of bilateral vagotomy

In another group of cats, the responses to intravenous prostaglandin F$_{2\alpha}$ were observed before and after vagotomy eliminated both the afferent and efferent

Table 1 Effect of prostaglandin F$_{2\alpha}$ on pulmonary arterial pressure, mean systemic arterial pressure and heart rate

	0.1 µg/kg	*0.3 µg/kg*	*1.0 µg/kg*	*3.0 µg/kg*	*9.0 µg/kg*
Pulmonary arterial pressure (mmHg)					
Initial level	18.5 ± 1.8	17.5 ± 1.2	17.8 ± 1.1	16.8 ± 1.0	17.8 ± 0.7
Response	+9.2 ± 1.2	+16.5 ± 2.8	+21.8 ± 3.4	+28.3 ± 3.0	+30.2 ± 2.1
Duration (min)	2.5 ± 0.6	3.2 ± 0.7	4.2 ± 0.3	6.0 ± 0.7	7.5 ± 1.4
Mean systemic arterial pressure (mmHg)					
Initial level	158.0 ± 8.9	159.3 ± 7.0	155.3 ± 7.4	150.8 ± 9.6	150.8 ± 5.6
Response	+1.7 ± 2.8	−0.8 ± 2.3	−16.6 ± 8.6	−38.8 ± 10.2	−51.8 ± 10.1
Duration (min)	–	–	1.4 ± 0.2	3.3 ± 1.0	4.8 ± 1.0
Heart rate (beats/min)					
Initial level	195.4 ± 8.7	194.0 ± 7.4	192.0 ± 8.2	194.5 ± 8.1	199.0 ± 9.1
Response	−1.3 ± 0.6	+0.8 ± 0.8	−12.1 ± 6.0	−60.3 ± 16.9	−69.8 ± 18.6
Duration (min)	–	–	5.5 ± 0.9	7.6 ± 1.6	10.2 ± 2.7

Values are means ± s.e. of six experiments. All responses statistically significant ($P < 0.05$) except mean systemic arterial pressure and heart rate at 0.1 and 0.3 µg/kg.

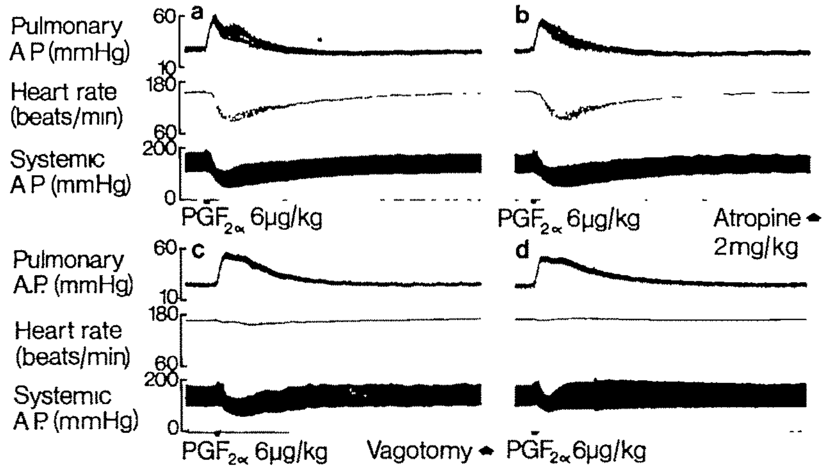


Figure 2 Effect of intravenous administration of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) on pulmonary arterial pressure, heart rate and systemic arterial pressure (a) and (b) are control responses Atropine (2 mg/kg) was administered between panels (b) and (c). Both vagi were sectioned prior to response seen in panel (d) Each panel represents 9 minutes. Time mark at 5 s intervals

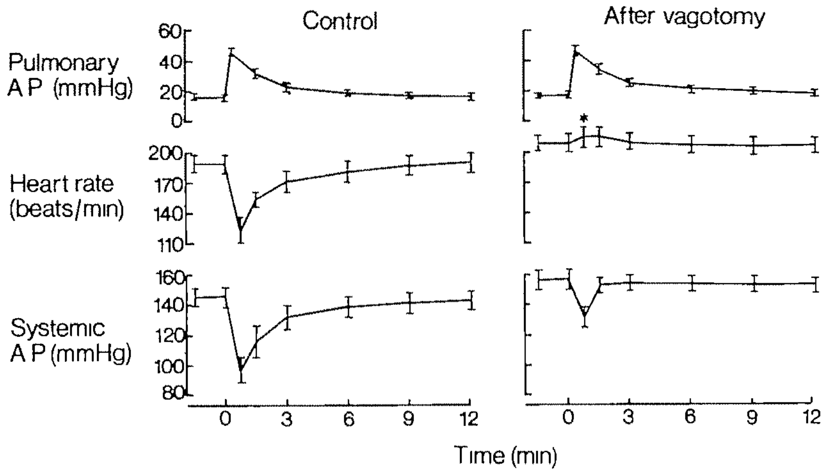


Figure 3 Composite representation of haemodynamic responses to maximally effective doses of prostaglandin F$_{2\alpha}$ (6–12 µg/kg, i v) before (left) and after bilateral vagotomy (right) Vertical bars represent ± s e. ($n=9$). All peak responses are significant ($P<0.01$) with the exception of the tachycardia following vagotomy * ($P<0.20$, NS)

vagal mechanisms. A typical response is shown in Figure 2d (in this case one of the previously atropine-treated animals was used). The composite responses of nine cats (not previously treated with atropine) are shown in Figure 3. As with atropine, bilateral vagotomy did not alter the increase in pulmonary arterial pressure. The prostaglandin F$_{2\alpha}$-induced bradycardia was abolished following vagotomy with most of these preparations exhibiting some degree of tachycardia ($P<0.20$, NS). As shown in Figure 3, vagotomy greatly reduced the magnitude of the depressor response and shortened the time of recovery to control levels. The blood pressure returned to basal levels in about 1.5–3 min in all of the vagotomized preparations.

Different injection sites

The previous results with atropine and vagotomy suggested that both afferent and efferent vagal

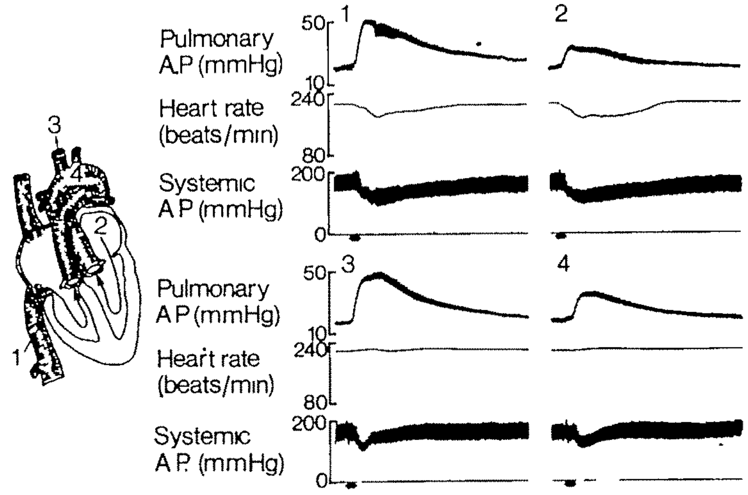


Figure 4 Cardiovascular actions of prostaglandin F$_{2\alpha}$ (4 μg/kg) injected (1) intravenously, (2) into the left atrium, (3) into the brachiocephalic artery and (4) into the aortic arch Numbers on drawing at left (1–4) correspond to responses seen in panels on right (1–4) Each panel represents 5 minutes

mechanisms might contribute to the bradycardia and hypotension associated with intravenous injections of prostaglandin F$_{2\alpha}$. With this in mind, an attempt was made to ascertain the loci or regions responsible for the bradycardia and hypotension produced by this agent. This was done by injection of submaximal doses (1–4 μg/kg) of prostaglandin F$_{2\alpha}$ at several locations. Depending on the sensitivity of the animal, these doses produced a moderate, submaximal bradycardia and systemic hypotension following intravenous injection.

Figure 4 illustrates the differential effects of prostaglandin F$_{2\alpha}$ administration in a typical experiment. Intravenous injection of prostaglandin F$_{2\alpha}$ (4 μg/kg) resulted in marked pulmonary constriction associated with a moderate bradycardia and hypotension (Figure 4–1). The same amount injected directly into the left atrium (Figure 4–2) resulted in less pulmonary constriction and a somewhat more extensive bradycardia. Injection into the brachiocephalic artery (Figure 4–3) or into the aortic arch at the level of the left subclavian artery (Figure 4–4) resulted in no change in heart rate and only a minimal depressor response which recovered rapidly. The constriction of the pulmonary vasculature was greater following brachiocephalic injection than was observed after administration into the aortic arch. This is probably due to the fact that prostaglandins are broken down by enzymes in the kidney and spleen but are not inactivated to any significant degree in the brain (Änggård, 1971; Nakano, Prancan & Moore, 1972).

Table 2 summarizes the haemodynamic responses to submaximal doses of prostaglandin F$_{2\alpha}$ following injection at these different sites in eight experiments. Note that the onset of bradycardia is much sooner after injection into the left atrium than after intravenous administration. In addition the magnitude and duration of the heart rate responses were greater following left atrial injections. The record shown in Figure 5 demonstrates these differences. In this example, 1 μg/kg administered intravenously resulted in the typical prostaglandin F$_{2\alpha}$-induced pulmonary arterial constriction, hypotension and bradycardia. The latency to onset was 38 s with the maximal bradycardia of 44 beats/min occurring in 70 seconds. The total duration of the response was 6.8 minutes. In contrast, the same dose (1 μg/kg) injected into the left atrium resulted in little or no pulmonary vasoconstriction. The heart rate slowed by 92 beats/min with a latency of onset of only 7 seconds. In this example the maximal effect was seen in 34 s and the duration of the response was 8.2 minutes.

A more specific localization is illustrated in Figure 6. In this example, prostaglandin F$_{2\alpha}$ (6 μg total dose = 1.8 μg/kg) was injected into the ascending aorta with the tip of the catheter just distal to the aortic valve (Figure 6a). This injection at the level of the exit of the coronary arteries resulted in a pronounced bradycardia associated with prolonged hypotension but only a minimal pulmonary arterial constriction. Administration of the same dose into the aortic arch (Figure 6b) produced no change in heart rate or systemic blood pressure. Control injections of saline were without effect at either site. In 3 additional experiments the results were consistent with the example shown in Figure 6. In two of these, the injection was made at the level of the aortic arch first, followed by injection at the level of the coronary arteries. Administration of pro-

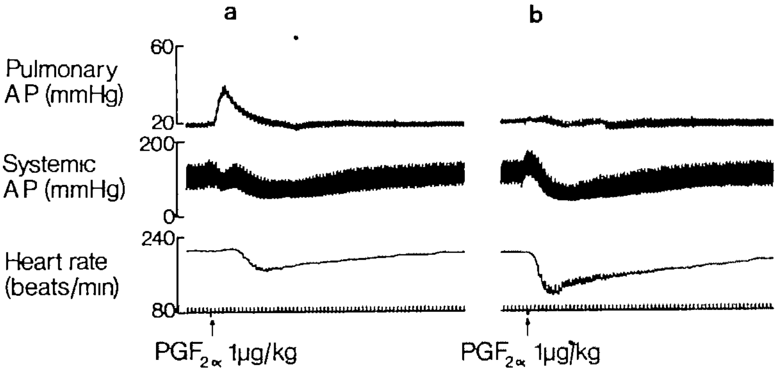


Figure 5 Comparison of the haemodynamic effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 1 μg/kg) injected intravenously (a) and into the left atrium (b). Note the shorter latency and greater magnitude of the bradycardia following the left atrial injection. Time mark is at 5 s intervals. Each panel represents 6 minutes.

staglandin $F_{2\alpha}$ (1–2 μg/kg) at the level of the coronary arteries resulted in a bradycardia of 52.2±5.8 beats/min in these four experiments. The mean latency was 13.5±4.1 s and the average duration was 7.7±2.1 minutes. In three of these experiments the hypotension resulting from prostalglandin $F_{2\alpha}$ injected near the coronary arteries occurred concomitantly with the bradycardia. The average decrease in blood pressure was 40.0±4.2 mmHg with a latency of 11.3±2.7 s and a duration of 7.6±2.2 minutes. The fourth cat had no change in blood pressure even though the heart rate decreased 46 beats/min (onset latency in this animal was 24 seconds). The same doses administered at the level of the aortic arch resulted in no change in blood pressure or heart rate in any of these experiments.

Table 2 Effect of prostaglandin $F_{2\alpha}$ (1–4 μg/kg) at different injection sites

	Intravenous	*Left atrium*	*Brachiocephalic artery*	*Aortic arch*
Pulmonary arterial pressure (mmHg)				
Initial level	18.1± 0.6	17.6± 0.5	16.3± 0.5	16.8± 0.5
Response	+24.3± 3.0	+11.6± 2.5	+17.0± 5.3	+11.3± 3.2
Onset (s)	5.4± 0.3	11.8± 1.6	10.4± 1.4	12.4± 4.1
Maximum (s)	25.4± 2.2	38.0± 6.7	31.5± 5.5	31.3± 2.7
Duration (min)	5.2± 0.7	4.5± 0.7	3.5± 1.2	3.5± 0.6
Mean systemic arterial pressure (mmHg)				
Initial level	149.0± 8.8	136.6± 7.8	135.5±14.2	133.5±11.2
Response	−37.6± 9.6	−38.8±11.4	−11.8±13.9	−0.4± 7.7
Onset (s)	11.2± 2.1	14.8± 2.1	–	–
Maximum (s)	54.5± 9.3	50.6± 6.2	–	–
Duration (min)	5.4± 1.4	6.1± 0.6	–	–
Heart rate (beats/min)				
Initial level	193.8± 9.1	192.6± 8.8	211.0± 5.5	200.3±11.6
Response*	−37.0± 8.9	−81.1±10.8	0 ± 1.6	−4.3± 2.5
Onset (s)	34.4± 5.8	8.8± 2.3	–	–
Maximum (s)	65.8±10.0	46.6± 4.3	–	–
Duration (min)	5.6± 1.1	8.1± 1.1	–	–

* Values represent means±s.e. of eight experiments. *n*=3 at 1 μg/kg; 1 at 2 μg/kg; 1 at 3 μg/kg; 3 at 4 μg/kg.

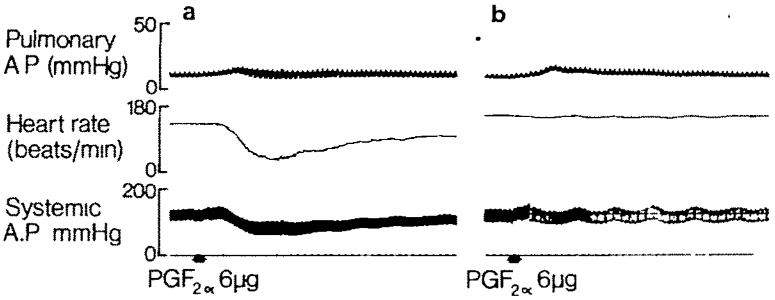


Figure 6 Effects of prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$, 6 μg total dose = 1.8 μg/kg) injected at the level of the origin of the coronary arteries (a) and into the ascending aorta distal to the exit of the coronary arteries (b). Note that the pronounced bradycardia and hypotension is observed only when this agent is able to enter the coronary circulation. Each panel represents 3.5 minutes

Discussion

The cardiovascular responses to prostaglandin F$_{2\alpha}$ are obviously extremely complex and appear to be composed of a variety of direct and reflex effects. In recent investigations (Koss & Nakano, 1973; Koss *et al.*, 1973a,b) we have shown that direct vasodilatation is the primary factor responsible for the depressor action of prostaglandin E$_1$ in the cat. In contrast, the direct vasodilator response following intra-arterial injection of prostaglandin F$_{2\alpha}$ was not only variable from one preparation to another but was much more transient than the overall hypotensive response following systemic administration. It was apparent in these previous studies that the depressor action of prostaglandin F$_{2\alpha}$ is due to a combination of haemodynamic factors other than direct vasodilatation. These factors include both a decrease in cardiac output resulting from pulmonary vascular constriction and an extensive delayed bradycardia which is abolished following vagotomy. The present experiments suggest that the hypotension and bradycardia produced by prostaglandin F$_{2\alpha}$ in the cat is mediated, at least in part, by reflex mechanisms. A moderate but consistent decrease in heart rate associated with the systemic depressor response was observed after atropine but not following vagotomy. This observation supports the contention that afferent as well as efferent vagal mechanisms are involved in this response.

In their original investigation concerning the cardiovascular actions of prostaglandins, Änggård & Bergström (1963) observed that intravenous injection of prostaglandin F$_{2\alpha}$ results in a decrease in the systemic blood pressure of the cat. They concluded that this overall effect might be partially caused by an increase in pulmonary vascular resistance and cardiac slowing, and only to a minor extent to a direct vascular action. In addition, they reported that the bradycardia was delayed in onset (10–30 s) and could be abolished by vagotomy. Several years later, Horton & Main (1965) observed that both prostaglandin E$_1$ and F$_{2\alpha}$ increased blood flow through skeletal muscle and lowered arterial blood pressure in the cat. In these studies prostaglandin F$_{2\alpha}$ was found to be about one-tenth as active as E$_1$.

Änggård & Bergström (1963) suggested that the vagal activation induced by prostaglandin F$_{2\alpha}$ might be related to reflex effects elicited from pressure receptors in the lungs or from the right side of the heart. Earlier workers have described reflex hypotension and bradycardia due to increased pulmonary arterial pressure or to increased venous pressure activating receptors in the right heart (Aviado, Li, Kalow, Schmidt, Turnball, Peskin, Hess & Weiss, 1951; Dawes & Comroe, 1954). The present results do not support this hypothesis as injection of prostaglandin F$_{2\alpha}$ into the ascending aorta at the level of the origin of the coronary arteries continued to elicit an extensive bradycardia and hypotension without the concomitant increase in pulmonary arterial pressure. The observation that the reflex effects of prostaglandin F$_{2\alpha}$ were greater after injection into the left side of the heart than after intravenous administration, suggests that right atrial or right ventricular receptors are not primarily responsible for the reflex actions of this agent.

In previous experiments we were unable to demonstrate any direct brainstem action following direct injection of prostaglandin F$_{2\alpha}$ into the left vertebral artery (Koss *et al.*, 1973a). In the present study, doses of prostaglandin F$_{2\alpha}$ producing a moderate vagal activation when injected intravenously, were ineffective when injected into the brachiocephalic artery or into the aorta distal to the origin of the coronary arteries. These results suggest that this agent does not exert a major action on structures distal to the cardiopulmonary region. At these low dosage levels, prostaglandin F$_{2\alpha}$ does not appear to produce cardiovascular effects by acting directly on the carotid bodies, nodose ganglia or various mid- and forebrain structures. McQueen & Belmonte (1974) have recently reported that prostaglandin $F_{2\alpha}$ has no direct action upon carotid baroreceptors or chemoreceptors in the cat.

It is of interest that brachiocephalic injection results in pulmonary arterial constriction of almost the same magnitude as seen following intravenous administration. On the other hand, the pulmonary vascular response is reduced following injection of the same dose into the left heart or into the descending aorta. These results are consistent with the observation that prostaglandins are rapidly metabolized by enzymes concentrated in the kidney, lungs and spleen (Änggård, 1971) and are not inactivated to any significant degree following passage through the brain (Nakano *et al.*, 1972).

Following the original observations by von Bezold & Hirt in 1867, a large number of cardiovascular reflexes have been shown to be the result of activation of structures or receptors within the heart and lungs. Comprehensive reviews of these varied responses have been presented by Dawes & Comroe (1954) and more recently by Paintal (1973). As mentioned previously, it is unlikely that prostaglandin $F_{2\alpha}$ exerts its primary action in the lungs, either by the increase in vascular resistance or by a direct action on pulmonary chemoreceptors.

In the cat, the aortic bodies received their blood supply, at least in part, via the coronary arteries (Coleridge, Coleridge & Howe, 1967). However, chemical activation of the aortic bodies would be expected to produce the opposite effects of those observed in the present experiments. It has been demonstrated that, in both dogs and cats, chemical activation of the aortic bodies usually results in a rise in blood pressure and heart rate whereas activation of the carotid bodies results in bradycardia and hypotension (Comroe, 1939; Dawes & Comroe, 1954; Comroe &Mortimer, 1964; MacLeod & Scott, 1964; Daly & Ungar, 1966).

As early as 1915, Cramer demonstrated conclusively that small doses of veratrum viride activate vagal afferents and that both reflex bradycardia and reflex peripheral vasodilatation contribute to the overall hypotensive response. Subsequent investigators demonstrated that the reflex decrease in blood pressure and heart rate following injection of small doses of veratridine is due primarily to an action upon receptors in the heart (Dawes, 1947; Aviado, Pontius & Schmidt, 1949). More recent observations indicate that receptors in the cardiopulmonary region exert a continuous restraint on sympathetic outflow by means of afferent vagal fibres (Mancia, Donald & Shepherd, 1973). These studies suggest that a concomitant inhibition of sympathetic outflow may be a frequent result of pronounced afferent vagal activation.

One possible explanation for the action of prostaglandin $F_{2\alpha}$ is that this agent induces a coronary chemoreflex mechanism by virtue of its action on receptors perfused by the coronary circulation or by acting on structures located in the left heart. A similar action is not seen in the dog in that prostaglandin $F_{2\alpha}$ produces an overall pressor response (Nakano & Cole, 1969). 5-Hydroxytryptamine and phenyldiguanide also produce different cardiovascular responses in the dog and cat (Dawes & Comroe, 1954). However, if this mechanism is responsible for the action of prostaglandin $F_{2\alpha}$ it is more selective in that only the cardiac receptors appear to be activated by prostaglandin $F_{2\alpha}$. Both 5-hydroxytryptamine and phenyldiguanide produce a characteristic triad of reflex apnoea, bradycardia and hypotension by acting on many receptors including those in the lungs and nodose ganglia as well as in the heart (Jacobs & Comroe, 1971). It is not known whether larger doses of prostaglandin $F_{2\alpha}$ might also activate these other receptors.

This investigation was supported by grants from the Oklahoma Heart Association and the Tulsa Chapter of the American Heart Association.

References

ÄNGGÅRD, E. (1971). Studies on the analysis and metabolism of the prostaglandins. *Ann. N Y. Acad Sci.*, **180**, 200–217

ÄNGGÅRD, E & BERGSTROM, S. (1963). Biological effects of an unsaturated trihydroxy acid ($PGF_{2\alpha}$) from normal swine lung. *Acta physiol. scand.*, **58**, 1–12.

AVIADO, D M , Jr., PONTIUS, R.G. & SCHMIDT, C.F. (1949). The reflex respiratory and circulatory actions of veratridine on pulmonary, cardiac and carotid receptors. *J. Pharmac. exp. Ther.*, **97**, 420–431.

AVIADO, D.M., Jr, LI, T. H., KALOW, W., SCHMIDT, C.F., TURNBULL, G.L., PESKIN, G.W., HESS, M.E. & WEISS, A.J. (1951). Respiratory and circulatory reflexes from the perfused heart and pulmonary circulation of the dog. *Am. J. Physiol.*, **165**, 261–277.

BERGSTRÖM, S., CARLSON, L.A. & WEEKS, J.R (1968). The prostaglandins: A family of biologically active lipids. *Pharmac. Rev.*, **20**, 1–48.

COLERIDGE, H., COLERIDGE, J C.G. & HOWE, A. (1967). A search for pulmonary arterial chemoreceptors in the cat, with a comparison of the blood supply of the aortic bodies in the new-born and adult animal. *J. Physiol., Lond.*, **191**, 353–374.

COMROE, J H., Jr. (1939). The location and function of the chemoreceptors of the aorta. *Am. J. Physiol.*, **127**, 176–191.

COMROE, J H, Jr & MORTIMER, L (1964) The respiratory and cardiovascular responses of temporally separated aortic and carotid bodies to cyanide, nicotine, phenyldiquanide and serotonin. *J. Pharmac. exp. Ther.*, **146**, 33–41.

CRAMER, W. (1915). On the action of veratrum viride. *J. Pharmac. exp. Ther.*, **7**, 63–82.

DALY, M DeB. & UNGAR, A (1966). Comparison of the reflex responses elicited by stimulation of the separately perfused carotid and aortic body chemoreceptors in the dog. *J. Physiol, London.*, **182**, 379–403.

DAWES, G S (1947). Studies on veratrum alkaloids VII. Receptor areas in the coronary arteries and elsewhere as revealed by the use of veratridine. *J. Pharmac. exp Ther.*, **89**, 325–342.

DAWES G S & COMROE, J H, Jr (1954). Chemoreflexes from the heart and lungs. *Physiol. Rev.*, **34**, 167–201.

DUCHARME, D W, WEEKS, J R. & MONTGOMERY, R G. (1968) Studies on the mechanism of the hypertensive effect of prostaglandin F$_{2\alpha}$. *J Pharmac. exp. Ther.*, **160**, 1–10.

HORTON, E W. & MAIN, I H M (1965). A comparison of the actions of prostaglandins F$_{2\alpha}$ and E$_1$ on smooth muscle. *Br. J Pharmac.*, **24**. 470–476.

JACOBS, L & COMROE, J.H, Jr (1971). Reflex apnea, bradycardia, and hypotension produced by serotonin and phenyldiguanide acting on the nodose ganglia of the cat. *Circulation Res.*, **29**, 145–155.

KOSS, M C., GRAY, J W., DAVISON, M. & NAKANO, J. (1973a). Cardiovascular actions of prostaglandins E$_1$ and F$_{2\alpha}$ in the cat *Eur. J. Pharmac.*, **24**, 151–157.

KOSS, M C. & NAKANO, J (1973). Effects of prostaglandins E$_1$ and F$_{2\alpha}$ on the peripheral circulation in the cat *Proc. Soc. exp. Biol Med.*, **142**, 383–386.

KOSS, M C, RIEGER, J.A. & NAKANO, J. (1973b) Hemodynamic responses to prostaglandin F$_{2\alpha}$ in the cat: Selective blockade by meclofenamic acid. *Fedn. Proc.*, **32**, 787

MACLEOD, R D M & SCOTT, M J (1964). The heart rate responses to carotid body chemoreceptor stimulation in the cat. *J. Physiol., Lond*, **175**, 193–202.

MANCIA, G, DONALD, D.E & SHEPHERD, J T. (1973). Inhibition of adrenergic outflow to peripheral blood vessels by vagal afferents from the cardiopulmonary region in the dog. *Circulation Res.*, **33**, 713–721.

McQUEEN, D.S. & BELMONTE, C (1974). The effects of prostaglandins E$_2$, A$_2$ and F$_{2\alpha}$ on carotid baroreceptors and chemoreceptors. *Quart. J. exp. Physiol.*, **59**, 63–71.

NAKANO, J (1973). Cardiovascular Actions. In *The Prostaglandins*, ed., Ramwell, P.W., pp. 239–316. New York: Plenum Press.

NAKANO, J. & COLE, B. (1969). Effects of prostaglandins E$_1$ and F$_{2\alpha}$ on systemic, pulmonary, and splanchnic circulations in dogs. *Am. J Physiol.*, **217**, 222–227.

NAKANO, J., PRANCAN, A V & MOORE, S.E (1972). Metabolism of prostaglandin E$_1$ in the cerebral cortex and cerebellum of the dog and rat. *Brain Res.*, **39**, 545–548.

PAINTAL, A.S. (1973). Vagal sensory receptors and their reflex effects. *Physiol Rev.*, **53**, 159–227.

(Received March 11, 1975.
Revised October 6, 1975.)

Br. J. Pharmac. (1976), **56**, 255–261

THE EFFECTS OF INTRA-AMYGDALOID INJECTIONS OF 6-HYDROXYDOPAMINE ON AVOIDANCE RESPONDING IN RATS

J. ASHFORD[1] & B.J. JONES[2]
Portsmouth School of Pharmacy, King Henry I Street, Portsmouth PO1 2DZ

1 The effects of bilateral intra-amygdaloid injections of 6-hydroxydopamine (6-OHDA) on shuttle box avoidance acquisition, retention, and extinction, and passive avoidance acquisition were examined in rats.

2 Intra-amygdaloid 6-OHDA injections produced catecholamine depletion in and around the amygdalae but failed to reduce striatal dopamine concentrations.

3 Conditioned avoidance acquisition was markedly inhibited in 6-OHDA-treated rats whereas retention and extinction were only slightly impaired.

4 Passive avoidance acquisition was slightly but significantly improved in rats with amygdaloid 6-OHDA lesions.

5 Treated rats showed no motor abnormalities, they were not hypoactive in a photocell activity cage and they performed as well as controls on a rotating rod.

6 It is suggested that the conditioned avoidance acquisition deficit in rats with amygdaloid 6-OHDA lesions may be related to an impairment of associative learning rather than to perceptual or motor disturbances.

Introduction

Although the distribution of the main catecholamine-containing pathways in the brain is now well established (Fuxe, Hökfelt & Ungerstedt, 1970; Ungerstedt, 1971), a formidable amount of work remains to be undertaken to discover the functions of these pathways. We are particularly interested in the functions of the catecholaminergic projections to limbic areas in relation to behaviour and our present studies have been confined to the amygdala, an area rich in noradrenaline and dopamine-containing terminals (Ungerstedt, 1971).

Among the numerous aspects of behaviour modified by stimulation or ablation of the amygdala, avoidance responding has been studied extensively (see Richardson, 1973). Furthermore, it has been demonstrated recently that this particular parameter can be modified by specific lesions of catecholaminergic pathways in the brain produced by stereotactic injections of 6-hydroxydopamine (6-OHDA) (Fibiger, Phillips & Zis, 1974; Cooper, Howard, Grant, Smith & Breese, 1974). It was pertinent therefore, to study avoidance responding in rats with amygdaloid 6-OHDA lesions.

Accordingly, attempts were made to produce specific bilateral lesions of the catecholaminergic projections to the amygdala in rats by injection of 6-OHDA directly into the terminal areas. Subsequently, the rats were tested for acquisition, retention and extinction of a two-way shuttle box avoidance response and acquisition of a passive avoidance response.

Methods

Male hooded rats, weighing between 200 and 220 g at operation were used throughout these studies.

Intra-amygdaloid 6-hydroxydopamine injections

6-OHDA was injected bilaterally into the amygdalae using stereotactic surgery under ether anaesthesia. The coordinates used were determined histologically but were based on the atlas of König & Klippel (1963), viz: 4.6 mm anterior, 3.4 mm lateral and 2.3 mm below zero.

6-OHDA was dissolved in 0.9% w/v NaCl solution (saline) containing 0.2 mg/ml ascorbic acid and kept cool on ice; 4 μl of solution was injected from an 'Agla' micrometer syringe at the rate of 0.2 μl/10 seconds. Control animals received identical injections of the vehicle alone.

---

[1] Present address: Pharmacy Department, St. Luke's Hospital, Guildford, Surrey.

[2] Present address: Department of Pharmacology, Glaxo Research Limited, Greenford, Middx. UB6 0HE.

6-OHDA hydrobromide was obtained from Sigma Chemical Co. and doses quoted (4, 8 or 16 μg) refer to the base.

Monoamine determinations

Following completion of the behavioural experiments, rats were stunned, decapitated and their brains were removed and dissected rapidly at 0°C. The dissection procedure was modified from the method of Glowinski & Iversen (1966). From the area designated 'cortex' by these authors, the portions immediately adjacent to the hypothalamus and bounded laterally by the external capsule were designated 'amygdalae' and the portions immediately lateral to the 'amygdalae' were referred to as 'periamygdaloid cortex'. In addition, the rostral and caudal portions of the remaining 'cortex' were not pooled, but referred to as 'frontal cortex' and 'cortex' respectively. The 'hippocampus' was included in the 'midbrain' portion.

Immediately after dissection, tissues were frozen and stored in liquid air. After homogenization in 0.4 M perchloric acid, samples were centrifuged. Separation of the monoamines in the supernatant was achieved by ion-exchange chromatography (Atack & Magnusson, 1970). Noradrenaline was assayed by the method of Euler & Lishajko (1961), dopamine according to Atack (1973) and 5-hydroxytryptamine by the method of Maickel, Cox, Saillant & Miller (1968).

Conditioned avoidance experiments

A standard Ugo Basile rat shuttle box was used throughout the experiments.

(a) *Acquisition* Fourteen days after surgery, rats were given 5 sessions of 50 trials in the shuttle box, one per day for 5 consecutive days. Each trial consisted of a 5 s conditioned stimulus (CS) (light and buzzer), followed by a 10 s unconditioned stimulus (UCS) (0.5 mA footshock) during which the CS remained on. The sequence was terminated at any time by the rat crossing into the other compartment. Trial frequency was once every 30 seconds.

(b) *Retention* Before surgery, the rats were conditioned for 5 daily sessions in the shuttle box as above. Operations were performed one day after the last session and a further 14 days later, the animals were tested again for 5 daily sessions.

(c) *Extinction* Extinction of response was examined in the rats tested for acquisition and retention by subjecting them to a final 5 sessions in which the UCS was not applied.

Passive avoidance acquisition

The shuttle box was modified so that the whole of the grid floor was continuously electrified. The central partition was replaced by a wooden platform, 6 cm wide and 2 cm high, which spanned the width of the cage.

Fourteen days after operation, each rat was placed on the central platform and left in the apparatus for 3 minutes. The number of times the rats stepped on to the grid and the total time spent on the grid were recorded. A total of 5 consecutive daily sessions were given.

Determination of pain threshold

Immediately before the first conditioned avoidance acquisition trial, each rat was placed in the shuttle box and footshock was applied in increasing intensity, stepwise at 0.05 mA every 30 s, until the animals showed visible signs of discomfort. The current intensity at this point was considered to provide an estimate of pain threshold.

Food and water intake

Food and water were provided *ad lib* but the amounts removed by the rats were determined daily. The estimates of consumption were approximate, because spillage was not taken into account.

Spontaneous locomotor activity

Fourteen days after operation, each rat was placed in a photocell activity cage (Lehigh Valley Electronics). After a 10 min acclimatization period, the number of beam interruptions occurring in 20 min was recorded. This procedure was repeated daily for a further 4 days.

Performance on a rotating rod

The apparatus consisted of a motor-driven 6 cm diameter acrylic rod with a finely corrugated surface. The speed of rotation was controlled manually through a 'Variac' variable voltage transformer.

Fourteen days after operation, rats were placed individually on a partitioned 10 cm section of the rod. According to the principle of Jones & Roberts (1968), the speed of rotation was increased approximately linearly from rest to 28 rev/min in 40 s by predetermined manual adjustments of the speed control. The times at which the rats fell from the rod were recorded.

Results

Effects on brain monoamine concentrations

Intra-amygdaloid injections of 6-OHDA produced depletion of noradrenaline and dopamine in the amygdala but also in some of the surrounding areas

(Table 1). Confined but slight depletion was obtained with 4 µg 6-OHDA, less confined but greater depletion with 8 µg and widespread depletion was observed with 16 µg.

5-Hydroxytryptamine concentrations were not significantly altered by 6-OHDA.

Conditioned avoidance

6-OHDA produced a dose-related inhibition of conditioned avoidance acquisition (Figure 1), but retention of an acquired response was impaired only by the high dose (8 µg) (Figure 2). Regardless of whether the response had been fully acquired or not, 6-OHDA produced a slight but significant antagonism of response extinction (Figures 1 and 2). However, the effect did not appear to be dose-related.

None of the rats showed any overt signs of motor abnormality, nor was there any indication that their perception of the CS was impaired. However, the 6-OHDA-treated rats exhibited less fearfulness than controls on presentation of the CS during the early stages of acquisition.

Some experiments were performed on rats treated with 16 µg 6-OHDA and there was an almost complete absence of acquisition. However, since the biochemical data indicated that this treatment produced widespread catecholamine depletion, it was subsequently abandoned.

Passive avoidance acquisition

All rats fully acquired the response by the third session. Although there was no difference between the

Table 1 Effects of intra-amygdaloid 6-hydroxydopamine (6-OHDA) injections on monoamine concentrations in various parts of rat brain

	Controls	*4 µg 6-OHDA*	*8 µg 6-OHDA*	*16 µg 6-OHDA*
(a) Noradrenaline (n=*15*)				
Medulla	0.58±0.03	0.50±0.02	0.55±0.03	0.55±0.04
Cerebellum	0.28±0.04	0.27±0.03	0.29±0.03	0.30±0.04
Hypothalamus	3.14±0.12	2.65±0.14*	1.49±0.13*	1.06±0.16*
Amygdala	1.74±0.12	1.00±0.14*	0.75±0.03*	0.68±0.08*
Periamygdaloid cortex	0.91±0.04	0.49±0.09*	0.35±0.03*	0.30±0.05*
Striatum	0.37±0.03	0.35±0.03	0.36±0.03	0.23±0.06*
Midbrain	0.57±0.08	0.55±0.07	0.42±0.05	0.30±0.07
Frontal cortex	0.50±0.08	0.28±0.04*	0.25±0.02*	0.28±0.03*
Cortex	0.25±0.03	0.23±0.04	0.18±0.03*	0.16±0.05*
(b) Dopamine (n=*9*)				
Medulla	0.30±0.02	0.40±0.05	0.42±0.02	0.42±0.06
Cerebellum	0.31±0.03	0.29±0.03	0.29±0.02	0.27±0.03
Hypothalamus	1.65±0.15	1.43±0.11	1.25±0.15*	1.09±0.04*
Amygdala	1.60±0.21	1.10±0.09*	0.92±0.03*	0.80±0.02*
Periamygdaloid cortex	1.27±0.20	0.82±0.04*	0.78±0.08*	0.61±0.07*
Striatum	5.52±0.63	5.49±0.38	5.40±0.02	4.65±0.02*
Midbrain	0.50±0.03	0.52±0.10	0.56±0.08	0.35±0.07*
Frontal cortex	2.18±0.19	1.95±0.40	1.00±0.06*	0.80±0.05*
Cortex	0.84±0.05	0.81±0.16	0.82±0.03	0.59±0.02*
(c) 5-hydroxytryptamine (n=*15*)				
Medulla	0.66±0.05	0.69±0.02	0.60±0.04	0.63±0.04
Cerebellum	0.22±0.07	0.19±0.05	0.20±0.03	0.19±0.04
Hypothalamus	3.10±0.58	3.88±0.80	3.68±0.61	2.83±0.71
Amygdala	1.30±0.16	1.23±0.17	1.40±0.13	1.33±0.17
Periamygdaloid cortex	0.82±0.15	0.83±0.10	0.70±0.09	0.75±0.02
Striatum	1.65±0.12	1.32±0.12	1.53±0.11	1.50±0.12
Midbrain	1.05±0.21	1.26±0.18	1.18±0.09	1.00±0.26
Frontal cortex	0.74±0.08	0.73±0.04	0.78±0.04	0.70±0.05
Cortex	0.37±0.05	0.33±0.04	0.34±0.02	0.36±0.05

Figures are means (µg/g) ± s.e. mean.

* Significantly different from control ($P < 0.05$) as determined by *t* tests

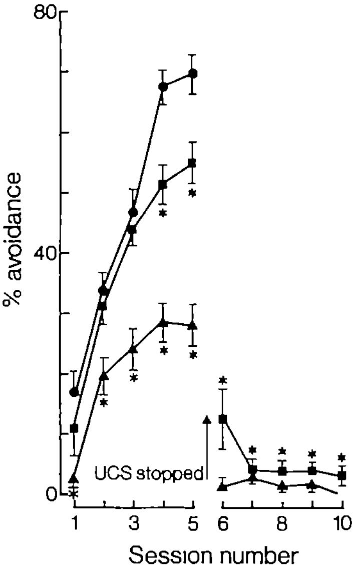


Figure 1 The acquisition and extinction of shuttle-box avoidance responses in rats after intra-amygdaloid injections of 6-hydroxydopamine (6-OHDA). Fourteen days after surgery, rats were given daily sessions (1–5) of 50 trials on 5 consecutive days. Subsequently, 5 sessions (6–10) were performed without the unconditioned stimulus (UCS). Extinction of response in controls was complete in session 6. (●) Control; (■) 4 µg 6-OHDA, (▲) 8 µg 6-OHDA. Points represent mean responses of 10 rats and the vertical bars show s.e mean *Significantly different from controls ($P < 0.05$) as determined by U tests.

performances of the controls and the 4 µg 6-OHDA-treated group, the rats treated with 8 µg 6-OHDA exhibited a significantly greater tendency to avoid the shock on the second session (Table 2).

Pain threshold

6-OHDA-treated animals were indistinguishable from controls with respect to the intensity of footshock required to elicit a pain response (Table 3).

Food and water intake

Temporary inhibition of both food and water intake was observed in 6-OHDA-treated animals, with a complete return to normal intake six days after operation (Table 4). Although no mortalities occurred in the reported experiments, it has been observed in subsequent experiments that a mortality rate of up to 20% may be encountered with 8 µg 6-OHDA unless forced feeding is instituted.

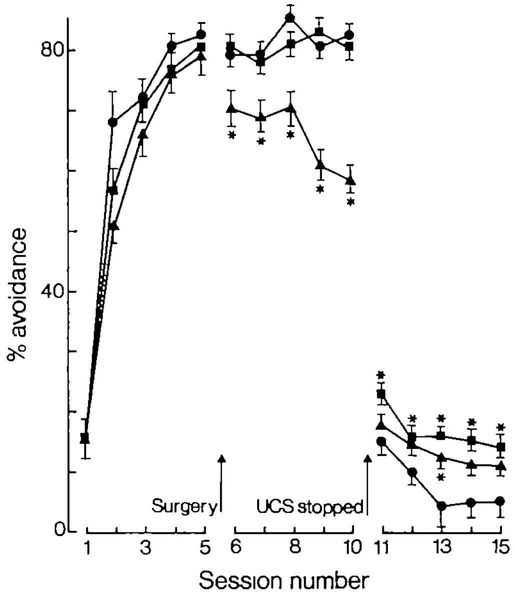


Figure 2 The retention and extinction of shuttle-box avoidance responses in rats after intra-amygdaloid injections of 6-hydroxydopamine (6-OHDA) Rats were given daily sessions (1–5) of 50 trials on 5 consecutive days. 6-OHDA was injected one day after session 5 and a further 14 days later, the rats were subjected to 5 more daily sessions (6–10). A final series of 5 sessions was performed without the unconditioned stimulus (UCS). (●) Control, (■) 4 µg 6-OHDA, (▲) 8 µg 6-OHDA Points represent mean responses of 10 rats and the vertical bars show s.e. mean *Significantly different from controls ($P < 0.05$) as determined by U tests.

Spontaneous locomotor activity

The data in Table 5 are presented as logarithms because this transformation was found to be most suitable for reducing heterogeneity of variance (Jones, Tolman & Roberts, 1970). The 6-OHDA-treated rats tended to be slightly more active than controls but on no occasion was there a statistically significant difference.

Performance on a rotating rod

There were no significant differences in the mean times at which controls and 6-OHDA-treated rats fell from the rotating rod (Table 6).

Table 2 The effects of intra-amygdaloid 6-hydroxydopamine (6-OHDA) injections on passive avoidance acquisition

(a) Mean number of shocks received (± s.e. mean) during 3 min

Session number	*Controls (n=15)*	*4 µg 6-OHDA (n=15)*	*8 µg 6-OHDA (n=14)*
1	11.1±1.7	11.6±1.2	8.6±0.9
2	3.6±1.1	4.4±1.3	1 7±0.5*
3	0	0	0
4	0	0	0
5	0	0	0

(b) Mean duration of shock received during 3 min (s ± s e. mean)

Session number	*Controls*	*4 µg 6-OHDA*	*8 µg 6-OHDA*
1	126±29	147±21	102±19
2	13.9±5.8	16.0±7.2	3.0±2.4*
3	0	0	0
4	0	0	0
5	0	0	0

* Significantly different from controls ($P<0\ 05$) as determined by *t* tests.

Table 3 The effects of intra-amygdaloid 6-hydroxydopamine (6-OHDA) injections on pain threshold

Control	*4 µg 6-OHDA*	*8 µg 6-OHDA*
0.22±0 01	0 22±0.01	0 21 ±0.01

The figures are mean threshold footshock intensities in mA ± s.e. mean. $n=10$ in each case

Table 4 The effects of intra-amygdaloid injections of 6-hydroxydopamine (6-OHDA) on food and water intake

(a) Mean daily food consumption (g/kg bodyweight ± s.e. mean)

	A	B	C	D
Controls	105±5	91±4	107±2	109±3
4 µg 6-OHDA	104±4	73±5*	102±31	103±4
8 µg 6-OHDA	109±4	71±5*	92±3	108±2

(b) Mean daily water consumption (cm^3/kg bodyweight ± s.e. mean)

	A	B	C	D
Controls	145±8	164±7	162±6	143±3
4 µg 6-OHDA	136±7	126±3*	149±5	127±5
8 µg 6-OHDA	151±5	110±9*	156±3	137±3

A=consumption on days 1–5 before surgery; B=consumption on days 1–5 after surgery, C=consumption on days 6–10 after surgery, D=consumption on days 11–15 after surgery
$n=10$ in each case. *Significantly different from controls ($P<0.05$) as determined by *t* tests.

Table 5 The effects of intra-amygdaloid 6-hydroxydopamine (6-OHDA) injections on spontaneous locomotor activity

Session number	*Controls*	*4 µg 6-OHDA*	*8 µg 6-OHDA*
1	2.269±0.063	2.345±0.078	2.348±0.038
2	2.184±0.119	2.215±0.132	2.359±0.054
3	1.822±0.159	1.863±0.204	1.988±0.154
4	1.861±0.113	1.884±0.195	2.140±0.109
5	1.705±0.188	1.935±0.216	1.770±0.145

Figures are the mean numbers of beam interruptions (as logarithms)±s.e. occurring in 20 minutes. $n=20$ in each case.

Table 6 The effects of intra-amygdaloid 6-hydroxydopamine (6-OHDA) injections on performance on a rotating rod

	Mean time of falling from rod (s)±s.e mean
Controls	28.6±4.7
4 µg 6-OHDA	25.6±3.1
8 µg 6-OHDA	28.3±5.3

$n=10$ in each case.

Discussion

The catecholamine depletion following intra-amygdaloid injections of 6-OHDA extended to extra-amygdaloid areas, so it was not possible to draw any conclusions about catecholamine function within the amygdala from our present results. Nevertheless, the extent of depletion was considerably less than would be obtained by intraventricular or intranigral injections of 6-OHDA.

The latter procedure is normally adopted for producing destruction of the nigro-striatal dopaminergic pathways, but amygdaloid projections are closely associated with these pathways (Ungerstedt, 1971) and would be expected to be destroyed also.

Recently, it was shown that bilateral intranigral injections of 6-OHDA impaired the acquisition of a one-way conditioned avoidance response (CAR) in rats (Fibiger *et al.*, 1974). This effect could be almost completely overcome by the administration of L-DOPA (Zis, Fibiger & Phillips, 1974), suggesting that depletion of striatal dopamine was possibly the most significant contributory factor to the CAR impairment. Support for this conclusion came from the work of Cooper, *et al.* (1974), who demonstrated pronounced CAR acquisition impairment produced by either destruction of the nigro-striatal pathways or intracaudate 6-OHDA injections.

We have demonstrated that impairment of CAR acquisition can be produced by bilateral intra-amygdaloid injections of 6-OHDA, which fail to produce striatal dopamine depletion. It is therefore possible that the deficit obtained by the above authors may be partly related to catecholamine depletion in extra-striatal areas.

Impairment of CAR acquisition could be due to a number of causes. Since our results were obtained in a two-way avoidance situation, we considered the possibility that the 6-OHDA-treated rats had an enhanced passive avoidance component of the shuttle response, i.e. they had an increased reluctance to return to the compartment in which they were shocked. We attempted to test this theory directly by studying a passive avoidance response. However, such a simple response is rapidly acquired by normal rats and improvements are therefore not readily discernible. Nevertheless, the rats treated with 8 µg 6-OHDA exhibited a slight but significant improvement in passive avoidance acquisition. This result cannot be considered definitive, but there is little doubt that 6-OHDA-treated rats did not have a passive avoidance deficit, indicating that there was no general learning impairment. Similar results were obtained by Cooper, Breese, Grant & Howard (1973) following intracisternal injection of 6-OHDA in rats. Their subjects failed to acquire either a one-way or a two-way active avoidance response yet were as capable as controls in learning a passive avoidance response. Such a profile of behavioural responses could simply reflect an impaired motor performance and this criticism is particularly relevant to studies in which the nigro-striatal dopamine pathways have been destroyed, since hypokinesia and rigidity may ensue (Jurna, Ruždić, Nell & Grossmann, 1972; Ungerstedt, Avemo, Avemo, Ljungberg & Ranje, 1973).

Our biochemical results indicated that intra-amygdaloid injections of 6-OHDA had spared the nigro-striatal pathways. Furthermore, 6-OHDA-treated rats showed no motor defects and they could not be differentiated from controls in spontaneous locomotor activity and rotating rod experiments.

The slight but significant inhibition of retention caused by intra-amygdaloid 6-OHDA injections was similar to the effect seen by Fibiger *et al.* (1974) following intranigral administration of 6-OHDA. The importance of this finding cannot be assessed because retention was studied two weeks after the last acquisition trial and a certain amount of re-acquisition may have been required. Thus, an acquisition deficit could readily account for the small changes observed.

An obvious possible cause of failure to acquire a CAR in the presence of an intact escape response could be an inability to perceive the CS. However, observation of rats with amygdaloid 6-OHDA lesions during presentation of the CS indicated that they perceived the CS although they appeared less fearful than controls. Lack of fearfulness is unlikely to be the direct cause of the acquisition failure, however, because lesioned rats exhibited sufficient fear of footshock to acquire rapidly a passive avoidance response. The apparent lack of fearfulness may merely reflect a failure to associate the CS with impending footshock. Indeed, it is possible that a disruption of the associative learning required to perform a CAR is the cause of the acquisition failure in rats with amygdaloid 6-OHDA lesions.

References

ATACK, C.V. (1973). The determination of dopamine by a modification of the dihydroxyindole fluorimetric assay. *Br J Pharmac.*, **48**, 699–714.

ATACK, C V & MAGNUSSON. T. (1970). Individual elution of noradrenaline (together with adrenaline), dopamine, 5-hydroxytryptamine and histamine from a single, strong cation exchange column, by means of mineral acid-organic solvent mixtures. *J. Pharm. Pharmac.*, **22**, 625–627.

COOPER, B.R, BREESE, G.R., GRANT, L D & HOWARD, J.L. (1973). Effects of 6-hydroxydopamine treatments on active avoidance responding: evidence for involvement of brain dopamine. *J. Pharmac. exp. Ther.*, **185**, 358–370.

COOPER, B.R., HOWARD, J L, GRANT, L.D., SMITH, R.D. & BREESE, G.R (1974). Alteration of avoidance and ingestive behaviour after destruction of central catecholamine pathways with 6-hydroxydopamine. *Pharmac. Biochem. Behav.*, **2**, 639–649.

EULER, U.S. VON & LISHAJKO, F. (1961). Improved technique for the fluorimetric estimation of catecholamines. *Acta physiol. scand.*, **51**, 348–355.

FIBIGER, H.C., PHILLIPS, A G. & ZIS, A.P. (1974). Deficits in instrumental responding after 6-hydroxydopamine lesions of the nigro-neostriatal dopaminergic projection. *Pharmac. Biochem. Behav.*, **2**, 87–96.

FUXE, K, HOKFELT, T. & UNGERSTEDT, U (1970). Morphological and functional aspects of central monoamine neurons. *Int. Rev. Neurobiol.*, **13**, 93–126.

GLOWINSKI, J & IVERSEN, L L (1966). Regional studies of catecholamines in the rat brain—I. The disposition of (^{3}H)-norepinephrine (^{3}H)-dopamine and (^{3}H)-DOPA in various regions of the brain. *J. Neurochem.*, **13**, 655–669.

JONES, B J & ROBERTS, D.J. (1968). The quantitative measurement of motor incoordination in naive mice using an accelerating rotarod. *J. Pharm. Pharmac.*, **20**, 302–304

JONES, B J, TOLMAN, B.D. & ROBERTS, D.J (1970). Studies on interactions involving antidepressive and other drugs with tetrabenazine and noradnamine on locomotor activity in mice, including details of the experimental design and statistical analysis. *Psychopharmacologia (Berl.)*, **18**, 288–299.

JURNA, I, RUŽDIĆ, N., NELL, T. & GROSSMAN, W (1972). The effect of α-methyl-*p*-tyrosine and substantia nigra lesions on spinal motor activity in the rat. *Eur. J. Pharmac.*, **20**, 341–350.

KÖNIG, J.F.R. & KLIPPEL, R A. (1963). *The rat brain. A stereotaxic atlas of the forebrain and lower parts of the brain stem.* Baltimore: Williams and Wilkins.

MAICKEL, R.P., COX, R.H, SAILLANT, J & MILLER, F.P. (1968). A method for the determination of serotonin and norepinephrine in discrete areas of rat brain. *Int. J. Neuropharmac.*, **7**, 275–281.

RICHARDSON, J S (1973). The amygdala: historical and functional analysis. *Acta Neurobiol Exp.*, **33**, 623–648.

UNGERSTEDT, U (1971). Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta. physiol. scand. Suppl.*, **367**, 1–148.

UNGERSTEDT, U., AVEMO, A., AVEMO, E., LJUNGBERG, T & RANJE, C (1973). Animal models of parkinsonism. *Adv. Neurol.*, **3**, 257–271.

ZIS, A.P, FIBIGER, H C. & PHILLIPS, A G. (1974). Reversal by L-DOPA of impaired learning due to destruction of the dopaminergic nigro-neostriatal projection *Science*, **185**, 960–962.

(Received July 14, 1975.
Revised November 12, 1975.)

Br. J. Pharmac. (1976), **56**, 263–269

EFFECTS OF AZAPERONE ON CARDIOVASCULAR AND RESPIRATORY FUNCTIONS IN THE HORSE

P. LEES & L. SERRANO[1]
Department of Physiology, Royal Veterinary College,
Hawkshead Lane, North Mymms, Hatfield, Herts

1 The butyrophenone tranquillizer, azaperone, was administered intramuscularly, at dose levels of 0.4 and 0.8 mg/kg, to ponies and its effects on cardiovascular and respiratory functions assessed.

2 Arterial blood pH, CO_2 tension ($PaCO_2$) and O_2 tension (PaO_2) remained relatively constant throughout the course of action of azaperone.

3 Azaperone did not modify plasma protein concentration but venous blood packed cell volume and haemoglobin concentration were reduced by 5 to 10% for at least 4 hours. These changes were probably caused by uptake of erythrocytes into the splenic reservoir.

4 Small increases in heart rate occurred for up to 60 min after administration of the drug, and this was followed by a slight bradycardia in some ponies.

5 Azaperone reduced mean arterial blood pressure (MAP) for at least 4 h, by which time its ataractic action was generally no longer apparent. The hypotension was caused, during the early phase of action at least, by a reduction in peripheral resistance, since cardiac output was increased slightly 20 min after its administration. Possible mechanisms underlying the cardiovascular changes are discussed.

6 In spite of reductions in arterial blood O_2 content and MAP produced by azaperone, it is likely that tissue oxygenation was adequate, since arterial blood lactate concentrations were not increased.

Introduction

In veterinary medicine tranquillizers are used extensively to quieten, calm and pre-medicate animals which, because of their individual or species temperament, may be less amenable to approach and handling than other animals or man. Drugs of the phenothiazine and butyrophenone groups, having proved particularly useful, have now largely superseded traditional sedatives, such as chloral hydrate, for use in many species. For example, azaperone is a relatively new butyrophenone derivative, which has been used in recent years in porcine (Marsboom & Symoens, 1968; Clarke, 1969; Symoens & van den Brande, 1969; Lang, 1970; Callear & van Gestel, 1971; Nienaber, 1972) and in equine (Roztočil, Němécek & Pavlica, 1971; Aitken & Sanford, 1972a,b; Ehmke, 1972; de Leglise, 1973; Serrano & Lees, 1976) medicine.

Previous reports on the actions of azaperone have been concerned primarily with its general properties and clinical efficacy. The present paper complements these studies by describing the pharmacological actions of azaperone on cardiovascular and respiratory functions in the horse. Possible mechanisms underlying the most profound action of azaperone, a moderate, long-lasting degree of hypotension, are considered.

Methods

Drugs, animals and experimental protocols

The drugs used were azaperone (Suicalm, Crown Chemical Co.) and heparin (Pularin, Evans Medical Co.).

The actions of azaperone were investigated in four series of experiments, using Welsh Mountain ponies of either sex, ranging in age from 3 to 5 years and in weight from 202 to 300 kg. No animal was used in more than three of the four series, and at least 10 weeks elapsed between each series. The sedative action of azaperone was assessed by means that have been described elsewhere (Serrano & Lees, 1976).

Both carotid arteries of each pony had been transposed to subcutaneous positions in the neck at least 6 months before the experiments (Tavernor, 1969). This facilitated cardiovascular measurements and the collection of samples of arterial blood. The ponies were trained to accept the recording procedures, and it is assumed, therefore, that all values were obtained from non-stressed animals, unless otherwise stated.

---

[1] Present address: National University of Colombia, Veterinary Faculty, Bogota, D.E., Colombia, S. America.

In the first experimental series, changes in heart rate were recorded in seven ponies before and at predetermined times after injecting azaperone, at a dose rate of 0.4 mg/kg, into the gluteal muscle. In the second series, intramuscular injections of azaperone were given to the same animals at the higher dose rate of 0.8 mg/kg and changes in heart rate again recorded. This dose level of azaperone always produced a satisfactory degree of ataraxy and it was used, therefore, in all subsequent experiments. In the third experimental series, six ponies each received an intramuscular injection of azaperone and the following variables were measured before and at predetermined times after its administration: arterial blood pH, CO_2 tension ($Pa\text{CO}_2$), O_2 tension ($Pa\text{O}_2$), lactate concentration and pyruvate concentration; venous blood haemoglobin (Hb) concentration and packed cell volume (p.c.v.) and venous plasma protein concentration; heart rate and mean arterial blood pressure (MAP).

In the fourth series of experiments six ponies each received an intramuscular injection of azaperone on three occasions at six-week intervals. Arterial blood pH, $Pa\text{CO}_2$, $Pa\text{O}_2$, MAP and cardiac output were measured both before and 20 min after injection, and further indices of cardiovascular function were derived from these measurements.

Cardiovascular measurements

Heart rate (beats/min) was measured from one of the standard limb leads of the ECG, recorded on a Devices M2 two-channel recorder. The rate was derived from the distance between ten consecutive QRS-complexes, using a chart speed of 10 or 25 mm/second. Arterial blood pressure was recorded from a carotid artery, into which a Braunula Number 1 catheter (Armour Pharmaceutical Co. Ltd.) had been inserted at least 1 h before the start of the experiment. The catheter was connected by a polythene tube containing heparinized saline (25 u/ml) to a Bell and Howell pressure transducer (type 4/327/L221). Recordings were made on the second channel of the Devices recorder. The transducer was placed 50 mm above the point of the shoulder in all ponies, because radiographic studies (Bell, Beltran & Price, personal communication) have demonstrated that the position of the right atrium is, on average, at this level in ponies of similar size to those used in this study. Mean values of systolic and diastolic pressure were derived from ten consecutive systoles and diastoles. MAP was calculated from the formula:

$$\text{MAP} = \text{Pd} + \frac{\text{Ps} - \text{Pd}}{3},$$

where Pd = mean diastolic pressure and Ps = mean systolic pressure (Folkow & Neil, 1971). Results are expressed in mmHg.

Cardiac output (CO) was determined by a dye dilution technique using indocyanine green dye (Cardiogreen; Hynson, Westcott and Dunning, Inc.) as described previously (Hillidge & Lees, 1975). Values are expressed in litres/minute. Left ventricular stroke volume (LVSV) was calculated by dividing CO by the simultaneously recorded heart rate. Left ventricular stroke work (LVSW) was determined as the product of MAP and LVSV, and expressed in kp m. Total peripheral resistance (TPR) was calculated from the formula:

$$\text{TPR} = \frac{\text{MAP} \times 1{,}334 \times 60}{\text{CO} \times 1{,}000}$$

Values of TPR are approximate, since the formula assumes that left ventricular end-diastolic pressure equals zero. The values are expressed as dyn s cm^{-5}.

Respiratory measurements

Samples of arterial blood for determining pH, $Pa\text{O}_2$ and $Pa\text{CO}_2$ were obtained from the Braunula catheter in the carotid artery. Each sample was collected anaerobically in a heparinized 10 ml glass syringe (5,000 u/ml). The Clark O_2 electrode (Radiometer type E 5046) and Severinghaus CO_2 electrode (Radiometer type E 5036) were mounted in thermostatted cells. Values of $Pa\text{O}_2$ and $Pa\text{CO}_2$, expressed in mmHg, were read on a pH meter (Radiometer pH 27) linked to a gas monitor (Radiometer pH A 927). Arterial blood pH was determined with the Radiometer pH meter 27 in conjunction with a microelectrode system (Radiometer type E 5021). Blood samples were either analysed immediately or stored on ice and read within 45 min of collection. Measurements were made at a known, constant electrode temperature within the range of 37 to 38°C. All readings were later corrected to the animal's rectal temperature at the time of sampling using a blood gas calculator (Severinghaus, 1966).

Analytical procedures

Samples of arterial blood for measurement of lactate and pyruvate concentrations (Hillidge & Lees, 1974) were obtained from the Braunula catheter in the carotid artery. Venous blood samples for duplicate determinations of p.c.v., Hb concentration and plasma total protein concentration were collected from a Braunula Number 2 catheter, which had been filled with heparin solution (5,000 u/ml) and inserted in a jugular vein on the day preceding the experiment. The samples were collected in 10 ml glass syringes, lubricated with heparin solution (5,000 u/ml).

Estimations of p.c.v. were made using a capillary tube microhaematocrit method (Hawksley and Sons

Ltd.). Blood Hb concentrations were measured by the cyanmethaemoglobin method of Drabkin with a Pye Unicam SP 600 spectrophotometer at a wavelength of 540 nm. Twenty mm^3 of well-mixed blood was added to 4 ml Drabkin's solution, mixed and allowed to stand at room temperature for at least 15 min before reading. Commercial cyanmethaemoglobin solutions (Diagnostic Reagents Ltd.) containing 3.0 and 18.0 g/100 ml were used as standards. Total protein concentrations in plasma were measured by the biuret method of Reinhold (1953), using an SP 600 spectrophotometer at a wavelength of 555 nm.

Statistical analyses

The significance of differences between mean values was assessed by the paired *t*-test at two levels, $P < 0.05$ and $P < 0.01$. Values quoted in the text are means together with s.e. means.

Results

Tranquillizing action of azaperone

The tranquillizing effect of azaperone was assessed in the first three series of experiments. In the first series, doses of 0.4 mg/kg produced levels of tranquillization that were classified as good or excellent in five of seven ponies, and as fair and slight, respectively, in the sixth and seventh animals. The degree of ataraxy was generally similar in the second and third series, when the higher dose level of 0.8 mg/kg was used, but a good or excellent response was obtained more consistently (twelve of thirteen instances). The onset of action was usually apparent within 10 min, becoming maximal after 10 to 70 minutes. The effects had always decreased by 2 h and usually were no longer apparent after 4 hours.

Heart rate

Azaperone produced small increases in heart rate for up to 60 min (Figure 1). The smallest degree of tachycardia occurred in response to a dose level of 0.4 mg/kg; at no time did the response reach significant levels. After 2 h, however, a significant degree of bradycardia was recorded ($P < 0.05$). In the second and third series of experiments a higher dose rate of azaperone (0.8 mg/kg) produced significant increases in heart rate (Figure 1), the greatest response occurring after 20 min and involving changes of 17% ($P < 0.05$) and 24% ($P < 0.01$), respectively, from the control levels. After the first hour, heart rate was reduced slightly below initial levels. At 4 h, for example, decreases of 11 and 10% were recorded in the second and third series, respectively, but these changes were not significantly different from the controls.

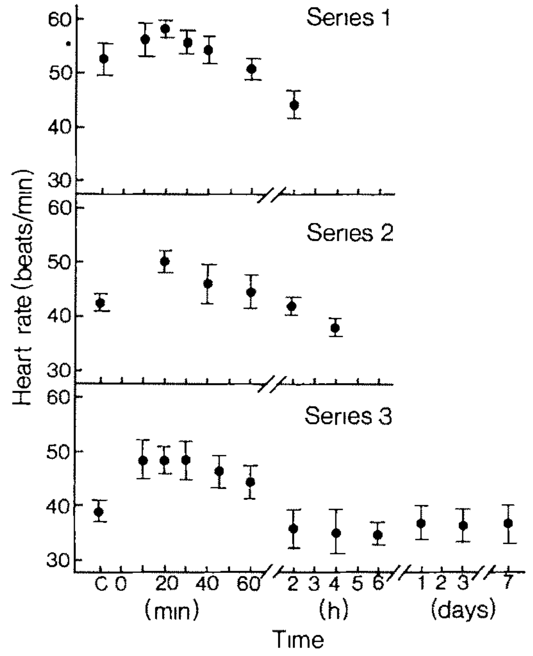


Figure 1 Effect of azaperone injected intramuscularly at zero time on heart rate of ponies C = control heart rates measured 5 to 15 min before administration of azaperone. Each point represents the mean value for seven animals each receiving 0.4 mg/kg (series 1), seven animals each receiving 0.8 mg/kg (series 2); and six animals each receiving 0.8 mg/kg (series 3). Vertical bars indicate s.e mean.

Mean arterial blood pressure

MAP was reduced following the administration of azaperone at a dose rate of 0.8 mg/kg (Figure 2). Pressures were reduced by 30 to 38% from the control level for up to 4 h ($P < 0.01$ at all times), the greatest decrease being recorded at one hour. After the first hour MAP increased slightly from the minimal level, but even after 4 h the pressure was still 35 mmHg less than the control value.

Cardiac output and total peripheral resistance

Since the degree of hypotension produced by azaperone was relatively constant between 10 and 120 min, while the tachycardia was maximal at 20 min, measurements of CO, MAP and TPR were obtained before and 20 min after injecting azaperone (0.8 mg/kg) in the next series of experiments. Azaperone was administered to each of six ponies on three occasions to provide average results from eighteen estimations. The mean control value of CO for the eighteen determinations was 23.1 ± 1.4

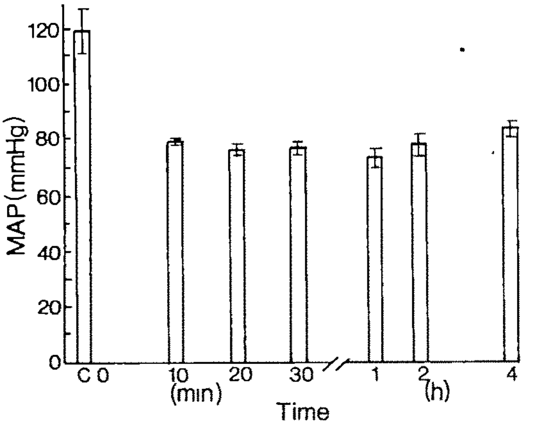


Figure 2 Effect of azaperone (0.8 mg/kg) injected intramuscularly at zero time on mean arterial blood pressure (MAP). C = control pressure recorded 5 to 15 min before administration of azaperone. Each column represents the mean value for five animals. Vertical bars indicate s e mean.

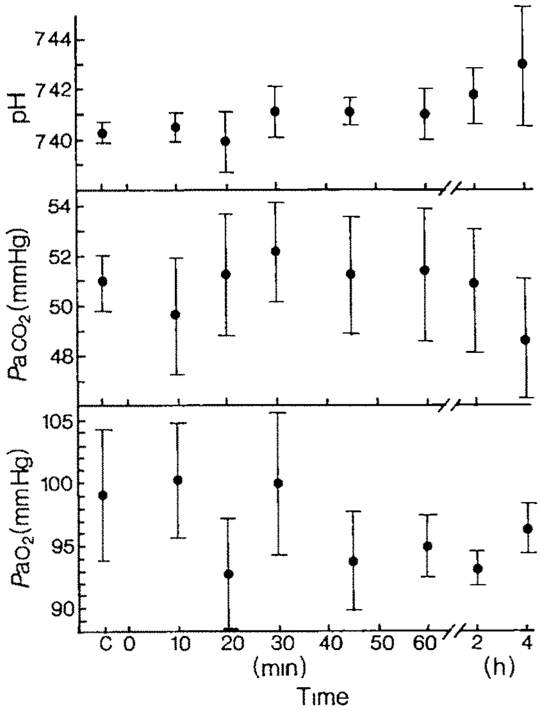


Figure 3 Effect of azaperone (0.8 mg/kg) injected intramuscularly at zero time on arterial blood pH, PaCO$_2$ and PaO$_2$. C = control values obtained 5 to 15 min before administration of azaperone Each point represents the mean value for five animals. Vertical bars indicate s e. mean.

1 min^{-1}, corresponding to 88.3 ± 4.0 ml kg^{-1} min^{-1}. Twenty minutes after injecting azaperone the mean CO was increased by 4% to 23.9 ± 1.8 l min^{-1} (91.9 ± 6.1 ml kg^{-1} min^{-1}). This change was not significant. Since MAP was decreased to a moderate extent, while CO was almost unchanged, TPR was reduced by 30% from 457 ± 34 to 319 ± 24 dyn s cm^{-5}. Further calculations revealed that LVSV and LVSW were also reduced, from 0.52 ± 0.03 to 0.39 ± 0.04 l/beat and from 0.86 ± 0.05 to 0.54 ± 0.05 kp m, respectively.

Arterial blood pH, Paco$_2$ *and* Pao$_2$

The slight changes in pH and *P*aco$_2$ recorded up to 4 h after azaperone administration (0.8 mg/kg) were not statistically significant (Figure 3). The greatest changes in *P*aco$_2$ comprised a slight increase of 2.4% at 30 min and a small decrease of 4.6% 4 h after administering the drug. In the fourth series of experiments azaperone (0.8 mg/kg) was administered to each of six ponies on three occasions. For these eighteen measurements the mean *P*aco$_2$ was 47.8 ± 0.9 before and 47.3 ± 0.8 mmHg 20 min after the injection, a difference that was also insignificant.

In the third experimental series values of *P*ao$_2$ tended to fall after injecting azaperone but the changes were small and insignificant (Figure 3). Likewise, for the eighteen experiments in the fourth series, *P*ao$_2$ decreased from a mean control level of 98.5 ± 1.3 to 95.0 ± 2.3 mmHg in samples collected 20 min after azaperone administration. This small change was also insignificant.

Venous blood packed cell volume and haemoglobin concentration

Mean control values of p.c.v. and Hb concentration for six ponies were 30.3 ± 1.3 and 10.9 ± 0.5, respectively. Small reductions in both variables occurred (Figure 4) in response to injections of azaperone (0.8 mg/kg). At each time interval the changes in the two variables were proportionally similar, so that mean corpuscular Hb concentration was almost unchanged. The decreases in p.c.v. and Hb concentration were apparent 10 min after injecting azaperone and maximal after 2 h, at which time both were reduced by 12% from the control levels. The absolute values at 10 min and 2 h, respectively, were 29.2 ± 1.0 and 26.8 ± 1.3 (p.c.v.) and 10.5 ± 0.4 and 9.7 ± 0.5 (Hb concentration). All values recorded between 30 min and 4 h were 8 to 12% less than the controls. Small increases in both variables occurred at 24 h and 3 days but they had returned to normal levels again by 7 days.

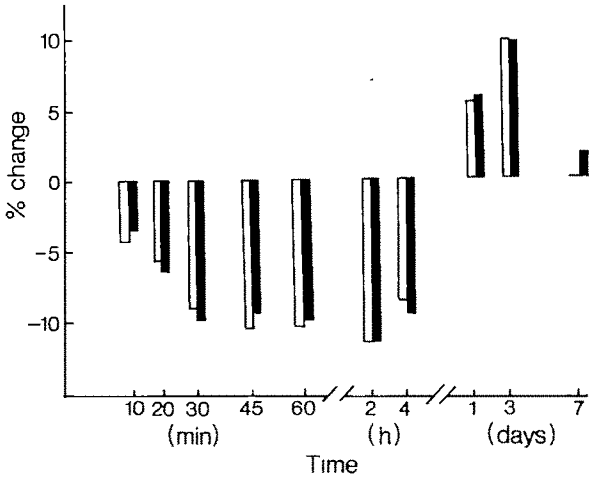


Figure 4 Percentage changes in jugular venous blood packed cell volume (closed columns) and haemoglobin concentration (open columns) following the intramuscular injection of azaperone (0.8 mg/kg) at zero time. Each column represents the mean change for six animals

Plasma protein concentration

Plasma protein concentration was affected only slightly by the administration of doses of 0.8 mg/kg azaperone (Table 1). The greatest change occurred 2 h after its administration, when the mean concentration was reduced by 1.3%. However, this was not significant at the 95% probability level.

Blood lactate and pyruvate concentrations

Concentrations of lactate and pyruvate in arterial blood were measured in three ponies. Both values fell slightly following azaperone administration, although the changes were not statistically significant (Table 1).

Discussion

The effects of azaperone on respiratory function call for little comment, since the slight changes in $Pa\text{CO}_2$, $Pa\text{O}_2$ and arterial blood pH were not statistically significant. However, arterial blood O_2 content was decreased by approximately 10% because of a reduction of this magnitude in Hb concentration. Whole body tissue oxygenation was probably adequate nevertheless. When tissue glycolysis proceeds anaerobically, lactate production leads ultimately to rises in circulating lactate levels but arterial blood lactate concentrations were not altered in this study.

Azaperone produced several changes in cardiovascular function: p.c.v. and Hb concentration were reduced by 5 to 12% for at least 4 h; a small though significant degree of tachycardia occurred for approximately 60 min while a slight bradycardia was recorded between 2 and 6 h in some ponies; and blood pressure was reduced by 30 to 50 mmHg for at least 4 hours. The degree of hypotension was relatively constant between 10 min and 4 h, whereas the tachycardia was maximal after 20 minutes. It was of interest, therefore, to note that cardiac output was not significantly affected at this time, so that the fall in pressure was attributable entirely to a reduction in total peripheral resistance. The present experiments do not establish the cause of the latter effect. It could be due, in part, to a reduction in blood viscosity resulting from the fall in p.c.v. An approximate estimate of the contribution of the latter to the 30% fall in TPR occurring 20 min after azaperone administration was made; a figure of 7% was obtained, suggesting that the remaining 23% of the decrease at this time was produced by some other mechanism.

It is probable that a second cause of the decrease in TPR was a vasodilator action of azaperone and this could be mediated in several ways. Marsboom (1969, 1971) has described a protective effect of azaperone against lethal doses of noradrenaline in rats, and others have demonstrated postsynaptic α-receptor

Table 1 Effect of azaperone on venous plasma protein concentration and arterial blood lactate and pyruvate concentrations

	Control	*Time after injecting azaperone* *10*	*20*	*30* *min*	*45*	*60*	*2*	*4* *h*
Protein (g/100 ml)	8.56±0.37	8.57±0.35	8.63±0.38	8.66±0.37	8.59±0.43	8.66±0.45	8.45±0.31	8.62±0.34
Lactate (mg/100 ml)	10.5±3.3	8.9±2.8	8.8±2.1	9.3±2.0	9.0±2.1	9.6±2.0	9.1±2.1	10.5±2.1
Pyruvate (mg/100 ml)	0.67±0.15	0.63±0.13	0.58±0.11	0.53±0.14	0.58±0.13	0.60±0.14	0.55±0.10	0.53±0.08

Each value is the mean with s.e. mean for six animals (protein concentration) and for three animals (lactate and pyruvate concentrations). No significant changes from control values occurred.

blocking activity of the butyrophenone on isolated tissues (Hapke & Prigge, 1972; Niemegeers, van Neuten & Janssen, personal communication) and reversal of the pressor action of adrenaline by azaperone in dogs and cats (Hapke & Prigge, 1972). Moreover, unpublished findings from this laboratory indicate that similar doses of azaperone to those used in this study partially antagonize several effects of adrenaline in ponies (rise in MAP, hyperkalaemia and increases in p.c.v. and Hb concentration), mediated at least partially by α-adrenoceptors. From this evidence it seems possible that the hypotensive action of azaperone may be attributable in part to blockade of vascular α-receptors and a consequent reduction in vasomotor tone. However, this action of azaperone may not account wholly for its vasodepressor effect because a study of the actions in ponies of acepromazine (0.1 mg/kg), another tranquillizer with α-receptor blocking properties (Marsboom, 1971), indicates that while it is somewhat more effective than azaperone (0.8 mg/kg) in suppressing the pressor effect of adrenaline it is less effective as a hypotensive agent (Serrano & Lees, 1976). It is clear that further investigations are needed to establish the cause of azaperone-induced hypotension.

An α-adrenoceptor blocking action of azaperone could also explain the reductions in p.c.v. and Hb concentration reported in this study. Other tranquillizers, including chlorpromazine, propriopromazine and promethazine, with known α-receptor blocking properties, decrease p.c.v. in sheep (Turner & Hodgetts, 1960a,b), dogs (Collette & Meriwether, 1965) and horses (de Moor & van den Hende, 1968). This action has been attributed to relaxation of the splenic capsule, the consequent enlargement of the spleen leading to uptake of erythrocytes from the circulation. However, there is some doubt concerning this hypothesis, since it depends on the presence of a tonically active sympathetic discharge to the capsule of the spleen, and Shepherd (1968) has claimed that little or no such activity exists. Another possible explanation for the reductions in p.c.v. and Hb concentration is a haemodilutant effect arising from an altered pre/post-capillary resistance ratio and a net transfer of interstitial fluid into the vascular compartment. Such an action has been reported in man for drugs, including guanethidine and phenoxybenzamine, which act in different ways to depress vasomotor tone (Weil & Chissey, 1968). However, this mechanism probably does not account for the present findings. This is indicated by the absence of significant changes in plasma protein concentration throughout the course of action of azaperone, when proportionally similar changes in p.c.v. and protein concentration would have been predicted by this hypothesis.

The findings of Roztočil *et al.* (1971) differed from those of the present investigation, these authors reporting an absence of significant changes in heart rate in horses receiving doses of 1 mg/kg azaperone intramuscularly. The small though significant degree of tachycardia produced by azaperone (0.8 mg/kg) in this study may have resulted indirectly from the hypotensive action of the drug through decreased sinus and aortic baroreceptor stimulation and a consequent change in the balance of sympathetic and parasympathetic nervous control of heart rate. However, the hypotension lasted for at least 4 h, whereas the tachycardia was usually no longer apparent after 1 h, suggesting that the reflex effect is overridden by some direct or indirect action to reduce heart rate. The mechanism of the secondary bradycardia which sometimes occurred is unclear. In any event it is of doubtful importance, since it attained a significant level only in the first series of experiments. This series was the only one to be carried out in an environment to which the ponies were unaccustomed. The mild apprehension that this might have caused could explain both the relatively high control heart rate (52 ± 2.7 beats/min) and the reduction in rate following azaperone administration.

This work was supported by the Horserace Betting Levy Board and L. Serrano received a grant from F.A.O. We are indebted to Mr F.C. Allsup, Miss J. Mozley and Mr D. Woulds for skilled technical assistance, Mrs I. Surridge for typing the manuscript and Crown Chemical Co. for supplies of azaperone.

References

AITKEN, M.M. & SANFORD, J. (1972a). Effects of tranquillizers on tachycardia induced by adrenaline in the horse *Br. Vet. J.* **128**, vii–ix.

AITKEN, M.M & SANFORD, J. (1972b). Comparative assessment of tranquillizers in the horse. *Proc. Ass. Vet. Anaes. G.Br. and Ir.*, **3**, 20–28.

CALLEAR, J F F. & VAN GESTEL, J F E (1971). An analysis of the results of field experiments in pigs in the U.K. and Ireland with the sedative neuroleptic azaperone. *Vet. Rec.*, **89**, 453–458.

CLARKE, K W. (1969). Effect of azaperone on the blood pressure and pulmonary ventilation in pigs. *Vet Rec.*, **85**, 649–651.

COLLETTE, W L & MERIWETHER, W.F (1965). Some changes in the peripheral blood of dogs after administration of certain tranquillizers and narcotics. *Vet. Med. Small Anim. Clin.*, **60**, 1223–1226

EHMKE, J. (1972). Stresnil in kombination mit polamivet zur sedation und narkose-pramedikation beim pferd. *Dtsch. tierarztl. Wschr.*, **79**, 539–543.

FOLKOW, B & NEIL, E. (1971) Measurements of pressures, flows and volumes in the cardiovascular system. In

Circulation p. 75. New York. Oxford University Press.

HAPKE, V H J & PRIGGE, E (1972). Herz-und kreislaufwirkungen von azaperon (stresnil). *Dtsch. tierarztl. Wschr.*, **79**, 500–504.

HILLIDGE, C.J. & LEES, P. (1974). Studies of whole body oxygenation in conscious and anaesthetised horses. *Archs. int. Pharmacodyn. Ther.*, **210**, 333–346.

HILLIDGE, C.J & LEES, P. (1975). Cardiac output in conscious and anaesthetised horses. *Equine vet. J.*, **7**, 16–21.

LANG, E. (1970). The use of azaperone for pigs. *Berl. Munch. tierarztl. Wschr.*, **83**, 141–143.

DE LEGLISE, J (1973). Contribution a l'etude de l'anésthesie générale. Emploi de l'azaperone (R1929) en prémédication chez le cheval. *These. Ecole National d'Alfort.*

MARSBOOM, R. (1969). On the pharmacology of azaperone, a neuroleptic used for the restraint of wild animals. *Acta. Zool. Pathol. Antverpiensia*, **48**, 155–161.

MARSBOOM, R. (1971). The development and pharmacology of butyrophenones in human and veterinary medicine. *Proc. Ass. Vet. Anaesth. G.Br. and Ir.*, **2**, 81–98.

MARSBOOM, R & SYMOENS, J. (1968). Azaperone (R1929) as a sedative for pigs. *Neth. J. vet Sci.*, **1**, 124–131.

DE MOOR, A. & VAN DEN HENDE, C. (1968). Effect of proprionylpromazine, promethazine and atropine on packed cell volume and circulating red cell mass in horses and cattle. *Zentbl. Med. Vet.*, **15A**, 544–548.

NIENABER, J (1972). Use of azaperone in veterinary practice. *Inaugural Dissertation, Tierarztliche Hochschule.* pp. 1–44. Hannover.

REINHOLD, J G. (1953). *Standard methods of clinical chemistry.* ed. Reiner, M. Vol 1. pp. 88–89. New York: Academic Press.

ROZTOČIL, V., NĚMĚCEK, L. & PAVLICA, J (1971). Stresnil as a sedative in horses. *Vet. Med. (Praha)*, **16**, 613–619.

SERRANO, L & LEES, P (1976). The applied pharmacology of azaperone in ponies. *Res. Vet. Sci.*, (in press).

SEVERINGHAUS, J W (1966). Blood gas calculator. *J. appl. Physiol.*, **21**, 1108–1116.

SHEPHERD, J T (1968). Personal communication to FOLKOW, B. & NEIL, E. (1971). Gastrointestinal and liver circulations. In *Circulation* p. 483. New York: Oxford University Press.

SYMOENS, J. & VAN DEN BRANDE, M. (1969). Prevention and cure of aggressiveness in pigs using the sedative azaperone. *Vet. Rec.*, **85**, 64–67.

TAVERNOR, W.D (1969). Technique for the subcutaneous relocation of the common carotid artery in the horse. *Am. J. Vet. Res.*, **30**, 1881–1883.

TURNER, A.W. & HODGETTS, V.E. (1960a). The dynamic red cell storage function of the spleen in sheep. *Aust. J. exp. Biol. Med. Sci.*, **38**, 79–90.

TURNER, A W & HODGETTS, V.E. (1960b). Fall in haematocrit in sheep after treatment with chlorpromazine and dihydroergotamine. *Nature, Lond.*, **185**, 43–44.

WEIL, J V. & CHISSEY, C.A. (1968). Plasma volume expansion resulting from interference with adrenergic function in normal man. *Circulation*, **37**, 54–61.

(Received July 22, 1975.
Revised November 18, 1975.)

Br. J. Pharmac. (1976), **56**, 271–277

BLOOD PRESSURE RESPONSES TO ADRENAL FIELD STIMULATION AS A MEASURE OF ADRENAL CATECHOLAMINE RELEASE

D.E. CLARKE & W.M. ROMANYSHYN
Department of Pharmacology, Division of Autonomic Neurochemistry,
University of Houston, Houston, Texas 77004, U.S A

1 A new method for studying adrenal medullary release is described in pithed rats using field stimulation of an entire adrenal gland.

2 The increases in blood pressure in response to field stimulation of the gland consisted of an initial short, variable component; due to stimulation of adrenergic vasomotor neurones and a secondary, longer lasting component, due to medullary catecholamine release.

3 Removal of the initial component by drugs or cardiac/coeliac ganglionectomy did not affect the magnitude of the secondary pressor component.

4 The secondary pressor component was frequency-dependent, reproducible and stable with time. The extent of medullary catecholamine release could be assessed by comparing the blood pressure rise with those obtained after adrenaline injections.

5 The method appears to provide a reliable means by which adrenal medullary catecholamine release may be assessed without significant interference from vasomotor nerves.

Introduction

The systemic blood pressure rise in response to splanchnic nerve stimulation has been used frequently to assess the activity of pharmacological agents on adrenal catecholamine release. However, splanchnic nerve stimulation also evokes a neuronally mediated vasoconstrictor response in the splanchnic vascular bed which forms a considerable component of the blood pressure response in the rat. In adrenalectomized rats with grafts of adreno-cortical tissue (Carpi & Cartoni, 1968) the blood pressure responses to low frequency splanchnic nerve stimulation remained unaltered, while at higher frequencies, they were still 60 to 65% of that obtained in the intact control rats.

In the present work the blood pressure response to direct electrical stimulation of the whole adrenal gland of pithed rats was analyzed for its usefulness as an indicator of adrenomedullary hormone release without interference by vasoconstrictor neurones. The availability of such a method could be important for the evaluation of adrenomedullary function. Extensive biochemical evidence has accumulated concerning drug-induced and hormonal effects on enzymatic activity in the adrenal medulla (Thoenen, Mueller & Axelrod, 1969; Kirshner, 1969; Mueller, 1971; Stjärne, 1972; Wurtman, Pohorecky & Baliga, 1972; Dairman, 1973; Costa & Guidotti, 1973), but correlations with the ability of the medulla to release catecholamines have generally not been made.

Methods

Male albino Sprague-Dawley rats (200–250 g) were pretreated with atropine (1 mg/kg, i.p.) and anaesthetized with pentobarbitone sodium (60 mg/kg, i.p.). After insertion of a tracheal cannula, the animals were pithed, and maintained on artificial respiration (Phipps & Bird, Model 71216) as described previously (Clarke, 1970). Blood pressure was recorded from a carotid artery, with a Statham (P23Dc) pressure transducer and a Narco Bio-Systems (DMP4a) physiograph and heart rate was measured with a Narco Bio-Systems biotachometer coupler (7302). The right femoral vein was cannulated for the injection of drugs, and both heparin (500 units/kg) and tubocurarine (1 mg/kg) were administered. A transverse incision was made in the ventral abdominal wall, just below the thorax, and the viscera were carefully retracted in order to expose the left adrenal gland. All bleeding was stopped with a cautery and the exposed liver and intestine were covered with saline-soaked gauze pads. The connective tissue and excessive fat surrounding the adrenal gland were cautiously teased away, avoiding damage to the vascular supply. Bipolar platinum electrodes (Palmer) were placed underneath the gland, which was held firmly on the electrodes by means of two ligatures previously passed through the periadrenal fat. The supramaximal voltage was determined for each preparation, and stimulations of 1 ms duration for 20 s were made over a range of

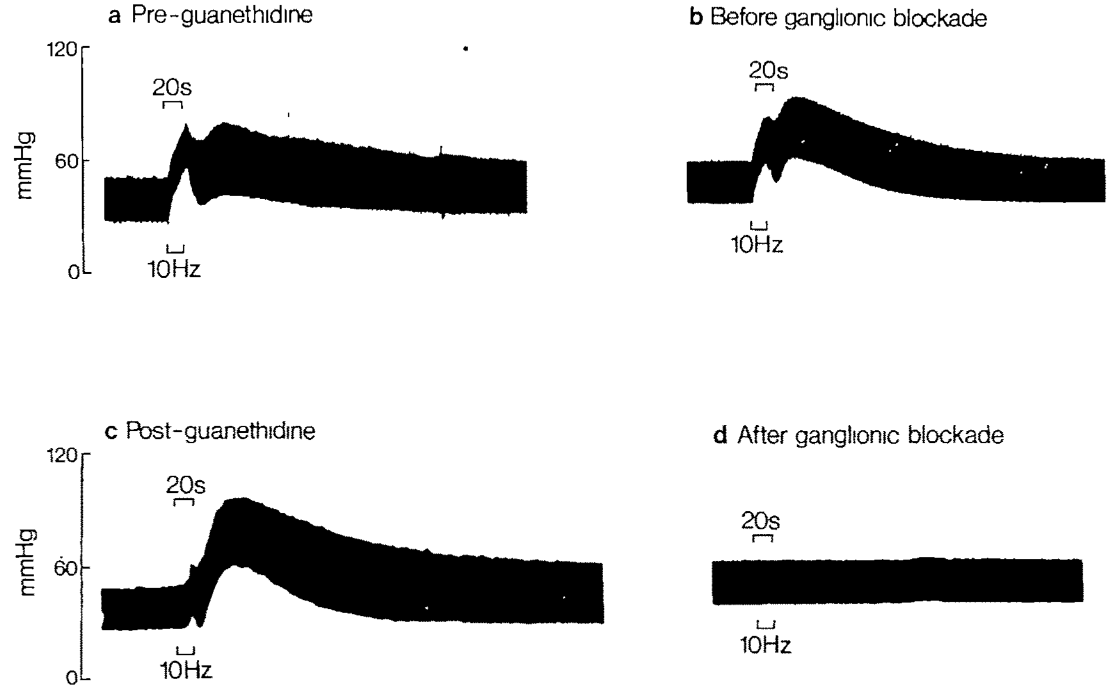


Figure 1 Blood pressure responses to field stimulation (10 Hz, 1 ms, 25 V for 20 s) of the entire left adrenal gland obtained in two pithed, atropine-(1 mg/kg, i p) and tubocurarine-(1 mg/kg, i.v) treated, rats (a) and (b) Controls, (c) guanethidine (5 mg/kg, i v) 2 h previously, (d) chlorisondamine (2 mg/kg, i.v) 10 min before stimulation

frequencies (0.5 to 20 Hz), with a Grass Instruments (SD5) stimulator. The peak rise in systolic blood pressure following stimulation, or adrenaline injection was measured.

Adrenal demedullated and sham-operated control rats were obtained from Zivic-Miller Laboratories, Inc. (Allison Park, PA). The animals were kept at 22°C on a diet of pellets (Purina Laboratory Chow) and given 0.9% w/v NaCl solution (saline) as the sole drinking fluid.

Drugs

The following drugs were used: adrenaline hydrochloride (Parke, Davis & Co.); chlorisondamine (Ciba); hexamethonium bromide (Sigma); guanethidine monosulphate (Ciba); reserpine (Ciba); 6-hydroxydopamine hydrobromide as base (Regis); tetracaine hydrochloride (Winthrop); procaine hydrochloride (Mallinckrodt); pentobarbitone sodium (Sigma); atropine sulphate (Mallinckrodt); tubocurarine chloride (Squibb); (±)-propranolol hydrochloride (Ayerst); phentolamine mesylate (Ciba); physostigmine salicylate (Mallinckrodt); sodium heparin (Riker); edrophonium chloride (Roche); bethanidine sulphate (Burroughs Wellcome); neostigmine bromide (Sigma).

Results

Figure 1 illustrates blood pressure responses following stimulation of the entire left adrenal gland of the pithed rat. Panels a and b show that a biphasic response was obtained. The magnitude of the initial pressor response was found to vary from one experiment to another. In fact, Figure 1a and 1b illustrates two of the larger responses obtained. In some experiments the response was very slight (2 to 5 mmHg), but it was always present. The amplitude of the second component was reproducible and longer lasting. Figure 1c shows that the injection of guanethidine (5 mg/kg, i.v.) diminished the initial component thus demonstrating the involvement of adrenergic neurones in this phase of the total response. Ganglionic blockade with chlorisondamine (2 mg/kg, i.v.) abolished both components (Figure 1d). This indicates the pre-synaptic nature of the total response. In order to check that the dose of chlorisondamine used did not

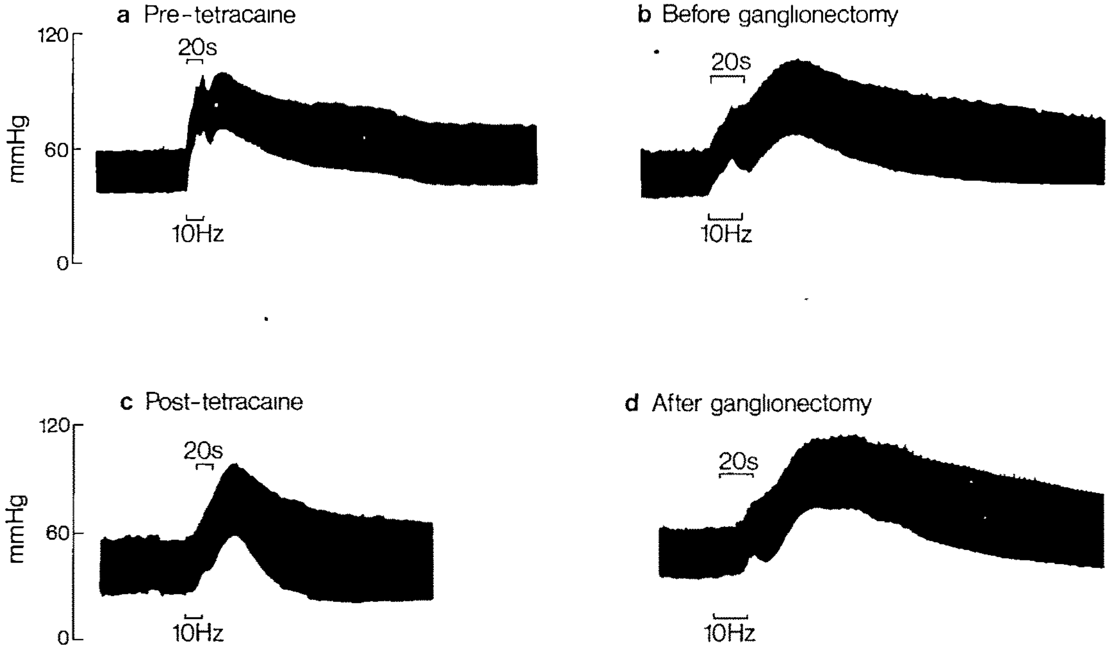


Figure 2 Blood pressure responses to adrenal field stimulation (10 Hz, 1 ms, 20 V for 20 s) of the entire left adrenal gland obtained in two pithed, atropine-(1 mg/kg, i.p.) and tubocurarine-(1 mg/kg, i v) treated, rats. (a) and (b) Controls, (c) after local application of tetracaine to the region of the cardiac and coeliac ganglia, (d) after surgical removal of the ganglia.

block postganglionic sympathetic fibres, the adrenergic innervation to the mesenteric vasculature was stimulated distal to the coeliac ganglion. Chlorisondamine failed to attenuate the resulting rise in systemic blood pressure.

Heart rate increases were measured in some experiments. Tachycardia was found to correlate with the onset and progression of the secondary response, suggesting that this component probably represents adrenal catecholamine release.

Selective inhibition of the initial component was obtained following the local application of procaine or tetracaine to the region of the prevertebral ganglia or by surgical removal of the cardiac and coeliac ganglia (Figure 2). A comparison of the frequency-response curves to adrenal stimulation (Figure 3) shows that no

Table 1 The effect of various drugs upon the blood pressure response (second component) to field stimulation of the adrenal gland and to intravenous adrenaline

		Blood pressure response	
Drug	*Dose (mg/kg, i.v.)*	*Adrenal gland stimulation*	*Adrenaline injection (12.5 to 50 ng/300 g body wt.)*
Chlorisondamine	2.0	Blocked	No effect
Hexamethonium	2.0	Blocked	No effect
Phentolamine	2.0	Reversal	Reversal
Propranolol	1.0	Potentiated	Potentiated
Phentolamine + Propranolol	2.0 + 1.0	Blocked	Blocked
Desipramine	0.05	Potentiated	Potentiated
Cholinesterase inhibitors	0.01–2.0	No consistent effect	No effect

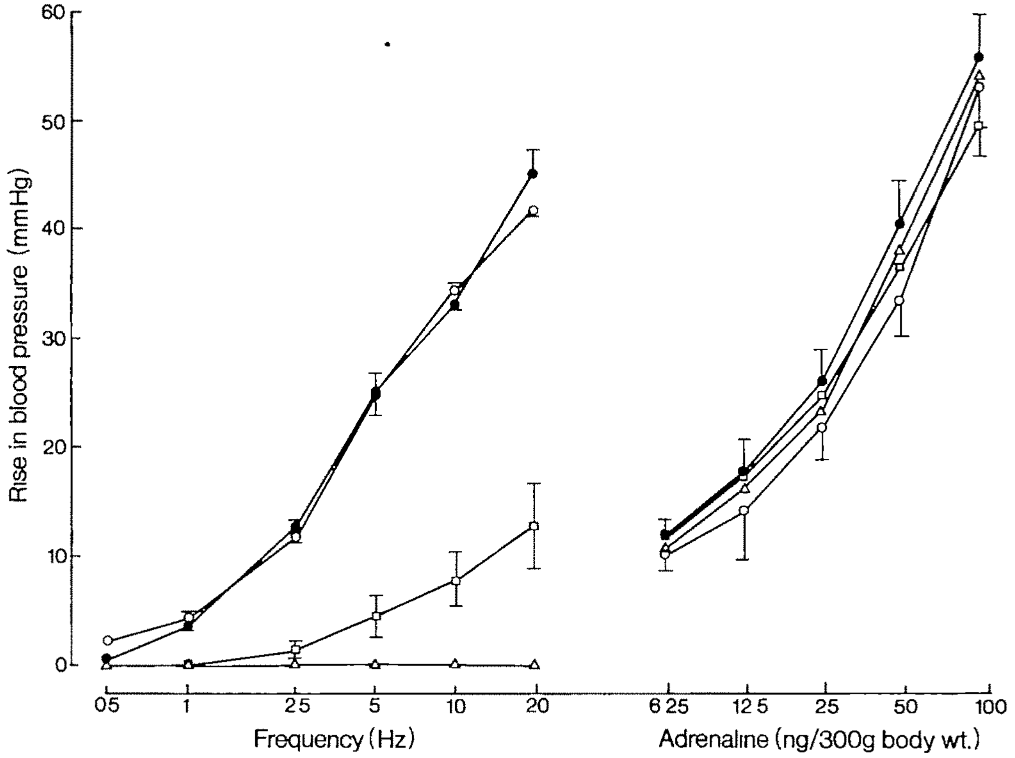


Figure 3 Relation between blood pressure increase (second component) and frequency of adrenal field stimulation (1 ms, 20 V for 20 s) or dose of intravenously injected adrenaline. (O) Control rats, (Δ) rats treated with chlorisondamine (2 mg/kg, i.v., $n=6$); (□) rats treated with reserpine (10 mg/kg, i.p. 18 h previously, $n=3$); (●) pooled data from rats in which the cardiac or coeliac ganglia were either removed or anaesthetized by local application of tetracaine All rats were pithed and pretreated with atropine and tubocurarine. Vertical lines show s.e. mean

significant differences are to be found between conditions where the initial component is present, or inhibited by the use of local anaesthetics or ganglionectomy. Thus the initial component, whether large or small, does not affect the amplitude of the secondary component.

Table 1 summarizes the effect of various drugs on the secondary component of the blood pressure response to adrenal field stimulation and injected adrenaline. Surprisingly, cholinesterase inhibition (with physostigmine, neostigmine or edrophonium) failed to increase the response, despite the indicated pre-synaptic origin. Stimulation of the adjacent body wall or left kidney also failed to evoke any pressor response but stimulation of the peri-adrenal fat after acute unilateral adrenalectomy, gave rise to the initial pressor response only. An identical result was obtained 1 to 2 days after bilateral adrenal demedullation. However, 3 to 10 days later no pressure response could be elicited by adrenal stimulation. The responses to intravenous adrenaline were unimpaired. Stimulation of the right adrenal gland is technically more difficult but in three experiments the blood pressure response obtained under these circumstances did not differ from that described for the contralateral adrenal.

Prolonged stimulation of the gland (10 to 15 min) was conducted. Submaximal frequencies gave rise to a stable hypertensive response with an accompanying tachycardia.

Figure 4 shows the quantitative reliability of the technique over the range of frequencies used. Dose-effect curves to intravenous adrenaline were made to enable a quantitative assay of the response to adrenal stimulation in terms of adrenaline release. Rats showed stable and reproducible responses with time and variation between animals fell within workable limits. This fact is well illustrated in the experiments in which reserpine pretreatment was given (10 mg/kg, i.p. 18 h previously). The frequency-response curve to

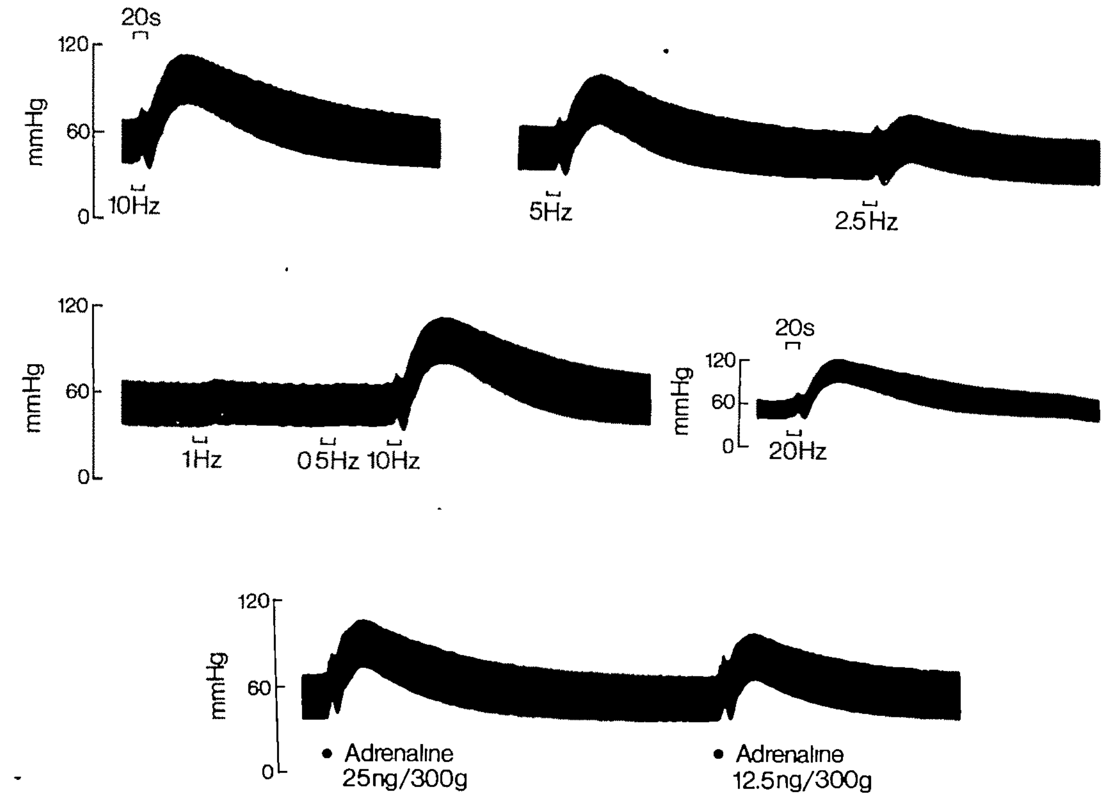


Figure 4 Effect of stimulation frequency on the blood pressure response (second component) to adrenal gland stimulation (1 ms, 25 V for 20 s) in a pithed, atropine-(1 mg/kg, i.p.) and tubocurarine-(1 mg/kg, i.v.) treated, rat given guanethidine (5 mg/kg, i.p., 2 h previously). Note the qualitative similarity to intravenous adrenaline (last two responses).

adrenal stimulation was depressed significantly whereas the dose-effect curve to adrenaline was not altered (Figure 3).

The effects of bethanidine and guanethidine pretreatment (both 5 mg/kg, i.p. 2 h previously), and 6-hydroxydopamine pretreatment (200 mg/kg, i.v. 18 to 22 h previously), were examined. Figure 5 shows that the frequency-response curves were shifted to the left; however, there was a corresponding shift in the dose-effect curves to intravenous adrenaline. These data seem to support the reported inability (Boura & Green, 1965; Thoenen & Oesch, 1973) of these agents to interfere with adrenal catecholamine release and show furthermore, that the extent of the evoked adrenal response can be expressed in quantities of adrenaline injected.

Discussion

Field stimulation of the entire adrenal gland elicited a rise in blood pressure, which consisted of a variable, short, initial component, and a longer lasting secondary component, the amplitude of the latter being dependent on the stimulation frequency. Guanethidine, and other adrenergic neurone blocking agents, abolished selectively the initial component, whereas chlorisondamine and hexamethonium inhibited both components. Presumably, electrical stimulation of the adrenal gland evoked a pre-synaptic excitation of cholinergic neurones leading to a depolarization of adrenergic vasoconstrictor fibres and of adrenal medullary chromaffin cells. These two effects would account for the biphasic blood pressure response. An initial vasoconstrictor component to splanchnic nerve stimulation has been reported in various species (De Vleeschhouwer, 1935; Nickerson & Goodman, 1947; Abercrombie & Davies, 1963), including the rat (Carpi & Cartoni, 1968). These responses would be expected to be fast in onset and short in duration, compared with a response resulting from medullary catecholamine release.

The initial component of the blood pressure response to adrenal field stimulation was shown to be

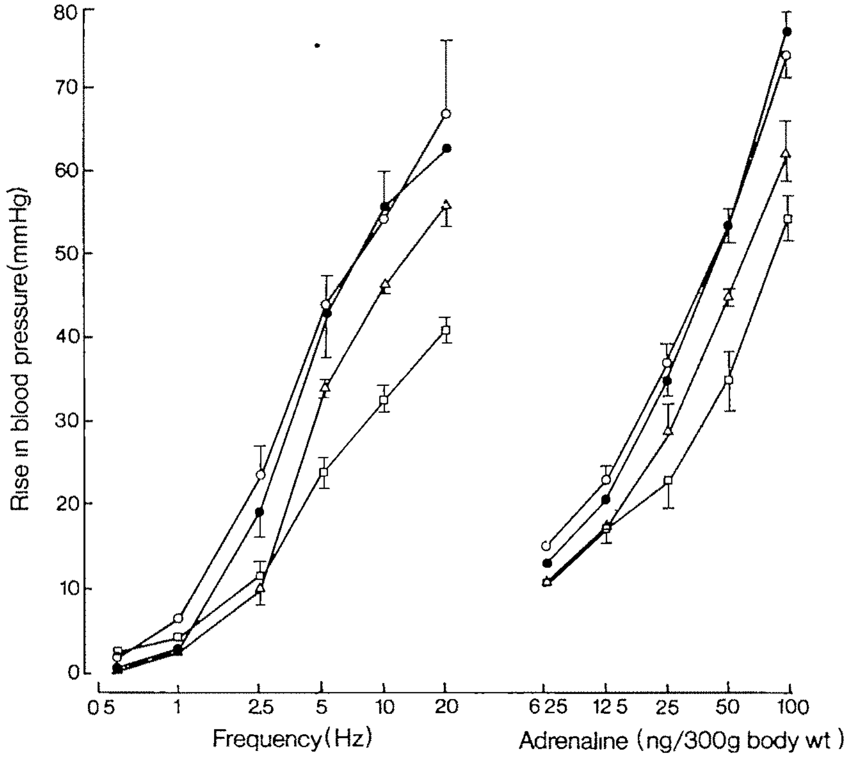


Figure 5 Relation between blood pressure increase (second component) and frequency of adrenal field stimulation (1 ms, 20 V for 20 s) or dose of intravenously injected adrenaline (□) Control rats; (○) rats pretreated with 6-hydroxydopamine (200 mg/kg, i.v. 18 h previously, $n=3$), (●) rats pretreated with guanethidine (20 mg/kg, i.p , 2 h previously, $n=7$), (△) rats pretreated with bethanidine (20 mg/kg, i p , 2 h previously, $n=4$) All rats were pithed and pretreated with atropine and tubocurarine Vertical lines show s e mean

of extra-adrenal origin and entirely dependent upon the functional presence of the cardiac and/or coeliac ganglia. The secondary component closely simulated the blood pressure response to an intravenous injection of adrenaline. It was accompanied by tachycardia and depended upon the presence of the adrenal medulla. Pharmacological characterization of the secondary component also suggests medullary catecholamine release, with adrenaline contributing to a major degree, as shown by the reversal of the response after α-receptor blockade.

The failure of cholinesterase inhibitors to increase the adrenal response to field stimulation cannot be explained at present. The response exhibited either no change or a decrease, irrespective of whether atropine was present or absent. Several possible explanations may be advanced, one possibility is that rat adrenal cholinesterase plays only a minor role in limiting the nicotinic effects of acetylcholine.

The absence of the initial vasoconstrictor component shortly after adrenal demedullation may be a reflection of progressive neuronal degeneration following surgery. This explanation would support the idea that adrenal field stimulation initiated antidromic impulses to the prevertebral ganglia via the splanchnic nerve, rather than inducing pre-ganglionic depolarization through current spread. However, in view of the high voltages used, this latter possibility cannot be ruled out. Some preparations exhibited supra-maximal responses at 4 or 8 V, but 20 to 25 V was always clearly supra-maximal. The encountered differences in the initial component between preparations may be related to variable electrode placement and/or to differing amounts of remaining periadrenal fat. Differing degrees of excitation of extra-adrenal splanchnic fibres is also likely to be involved.

The present experiments have shown that the initial vasoconstrictor response does not quantitatively influence the second component caused by the release of medullary amines. This represents a definite improvement over direct splanchnic nerve stimulation which, in the rat, has been shown to cause wide-spread neurogenic vasoconstriction (Carpi & Cartoni, 1968). Indeed, guanethidine reduced the response to splanchnic nerve stimulation indicating that the neurogenic vasoconstrictor component was so extensive as to preclude any precise quantitation of the catecholamines released from the adrenal medulla. The present findings show that the technique of adrenal field stimulation allows a more exact evaluation of adrenal catecholamine release. 6-Hydroxydopamine, guanethidine and bethanidine were found not to affect adrenal release as shown previously (Boura & Green, 1965; Thoenen & Oesch, 1973). The enhanced responses to adrenal stimulation found after these drugs were adequately accounted for by the increased responsiveness to injected adrenaline. In addition, responses to adrenaline were unchanged after reserpine but the impairment of adrenal medullary function was readily detectable.

The present paper describes an *in vivo* method for the quantitative assessment of the release of catecholamines from the adrenal medulla without interference from a neurogenic vasoconstrictor component. The method should be of use in studying the acute and chronic effects of drugs, hormones and induced pathological states (e.g. hypertension, hypophysectomy) upon the release of adrenal medullary amines.

This work was undertaken in partial fulfillment of the requirements for the degree of Ph.D at the University of Houston by W.M.R. Partial support for the work was obtained from Eli Lilly & Company, Indianapolis, Indiana 46206.

References

ABERCROMBIE, G G. & DAVIES, B.N (1963). The action of guanethidine with particular reference to the sympathetic nervous system *Br. J. Pharmac. Chemother.*, **20**, 171–177.

BOURA, A L.A & GREEN, A F. (1965) Adrenergic neurone blocking agents. *Ann Rev. Pharmac.*, **5**, 183–212.

CARPI, A. & CARTONI, C. (1968). Effects of guanethidine on the blood pressure response to splanchnic nerve stimulation in the rat: role of the adrenal medulla. *Br. J. Pharmac.*, **34**, 259–266.

CLARKE, D.E (1970). Restoration of tyramine responses by bretylium BW392C60, bethanidine and monoamine oxidase inhibitors in reserpine-treated rats. *Br. J. Pharmac.*, **38**, 1–11.

COSTA, E & GUIDOTTI, A (1973). The role of 3′,5′-cyclic adenosine monophosphate in the regulation of adrenal medullary function. In *New Concepts in Neurotransmitter Regulation*, ed. Mandell, A.J. pp. 135–152. New York: Plenum Press.

DAIRMAN, W (1973). Multiple measures of compensatory adaptation in catecholamine biosynthesis. In *New Concepts in Neurotransmitter Regulation*, ed. Mandell, A.J. pp 1–20. New York: Plenum Press.

DE VLEESCHHOUWER, G (1935). Au sujet de l'action du diethylaminomethyl-3-benzodioxane (F.883) et du piperidomethyl-3-benzodioxane (F.933) sur le système circulatoire. *Arch. Int. pharmacodyn. Thér.*, **50**, 251–295.

KIRSHNER, N (1969). Storage and secretion of adrenal catecholamines. In *Advances in Biochemical Psychopharmacology*, ed., Costa, E. & Greengard, P., Vol. 1. pp. 71–89. New York· Raven Press.

MUELLER, R.A. (1971). Effect of 6-hydroxydopamine on the synthesis and turnover of catecholamines and protein in the adrenal In *6-Hydroxydopamine and Catecholamine Neurons*, ed. Malmfors, T. & Thoenen, H. pp. 291–302 London: North-Holland.

NICKERSON, M & GOODMAN, L.S. (1947) Pharmacological properties of a new adrenergic blocking agent: N,N-dibensyl-chloroethylamine (Dibenamine). *J Phamac. exp. Ther.*, **89**, 167–185.

STJÄRNE, L. (1972). The synthesis, uptake and storage of catecholamines in the adrenal medulla. Effects of drugs In *Catecholamines*, ed. Blaschko, H. & Muscholl, E. pp. 231–269. Berlin: Springer-Verlag.

THOENEN, H., MUELLER, R.A. & AXELROD, J. (1969). Increased tyrosine hydroxylase activity after drug-induced alteration of sympathetic transmission. *Nature, Lond.*, **221**, 1264.

THOENEN, H & OESCH, F (1973). New enzyme synthesis as a long-term adaptation to increased transmitter utilization. In *New Concepts in Neurotransmitter Regulation*, ed. Mandell, A.J. pp. 33–52. New York: Plenum Press.

WURTMAN, R J., POHORECKY, L.A. & BALIGA, B.S. (1972). Adrenocortical control of the biosynthesis of epinephrine and proteins in the adrenal medulla. *Pharmac. Rev.*, **24**, 411–426.

(Received August 11, 1975.
Revised November 3, 1975.)

Br. J. Pharmac. (1976), **56**, 279–283

ON THE MECHANISM OF ACTION OF CLOZAPINE ON THE ADRENERGIC NEURONE

R.L. DORRIS & P.A. SHORE
Department of Pharmacology, University of Texas Health Science Center,
Dallas, Texas 75235, U S A

1 The antipsychotic drug, clozapine, lowered noradrenaline and metaraminol (MA) concentrations in the rat heart. This action was blocked by the presence of a ganglionic blocking drug.

2 Other α-adrenoceptor blocking drugs (phenoxybenzamine, phentolamine) did not significantly lower heart amine concentrations. An inhibitor of neuronal amine uptake (desipramine) caused only a slight lowering. The combination of phentolamine and desipramine showed considerable activity, and this action was blocked by ganglionic blockade.

3 Clozapine had little or no action in blocking amine uptake, yet greatly potentiated amine release caused by the phentolamine-desipramine combination.

4 Other antipsychotic drugs (haloperidol, chlorpromazine, thioridazine) or other agents (propranolol, atropine) did not share this action of clozapine.

5 Ganglionic blockade markedly delayed amine release induced by reserpine administration.

6 It is suggested that clozapine may have an incomplete reserpine-like effect specifically on the adrenergic neurone, facilitating impulse-induced amine release.

Introduction

The antipsychotic drug, clozapine, is of considerable current interest since it causes little or no extrapyramidal dysfunction (Stille, Lauener & Eichenberger, 1971; Angst, Bente, Berner, Heimann, Helmchen & Hippius, 1971; Angst, Jaenicke, Padrutt & Scharfetter, 1971; Stille & Hippius, 1971), even though the drug, like other antipsychotics, enhances dopamine turnover in the corpus striatum and limbic dopamine centres (Bartholini, Haefely, Jalfre, Keller & Pletscher, 1972; Andén & Stock, 1973). In addition to its other actions, clozapine exerts α-adrenoceptor blocking effects and shows considerable cholinolytic activity (Stille *et al.*, 1971). In fact, it has been postulated that the latter effect may be responsible for the lack of overt striatal dysfunction after clozapine administration (Andén & Stock, 1973; Snyder, Banerjee, Yamamura & Greenberg, 1974).

Clozapine has also been shown to cause a marked depletion of brain and spinal cord noradrenaline (NA), an action which is blocked by high doses of the α-adrenoceptor agonist, clonidine (Bartholini, Keller & Pletscher, 1973). It has been suggested therefore, that the NA-lowering effect of clozapine is due to marked α-adrenoceptor blockade followed by compensatory adrenergic neurone hyperactivity such as to release the adrenergic transmitter faster than synthesis can maintain normal concentrations. In agreement with this concept is the observation that clozapine depletes spinal cord NA cranial, but not caudal, to the site of a spinal transection (Bartholini *et al.*, 1973).

The present study examines the effect of clozapine on peripheral adrenergic neurones of the rat heart. It is shown that the drug lowers the concentration of heart NA and also releases the non-metabolizable false adrenergic transmitter, metaraminol (MA) by a nerve impulse-dependent mechanism. It is shown, however, that unlike other α-adrenoceptor blocking drugs or antipsychotic drugs tested, clozapine appears to exert a further action directly on the adrenergic neurone so as to facilitate impulse-dependent amine release.

Methods

Female Sprague Dawley rats (200-250 g) were used. In experiments in which the release of the false adrenergic transmitter, metaraminol (MA) was examined, (Shore, Busfield & Alpers, 1964; Crout, Alpers, Tatum & Shore, 1964) the amine was loaded in tissues by administration of the MA precursor, DL-α-methyl-*m*-tyrosine (100 mg/kg s.c., Regis Chemical Co.) 16-18 h before the experiments were started (Shore *et al.*, 1964).

Animals were killed by chloroform asphyxiation, hearts were removed and analyzed for NA or MA by fluorimetric methods described previously (Shore &

Alpers, 1964; Neff & Costa, 1966). Aqueous solutions of the following drugs were used: clozapine (Sandoz), haloperidol (McNeil Labs), phentolamine and chlorisondamine (Ciba), mecamylamine (Merck, Sharp-Dohme), (–)-metaraminol (Regis Chemical Co.), atropine (Sigma Chemical Co.), propranolol (ICI), chlorpromazine (Smith, Kline & French), and thioridazine (Sandoz). Phenoxybenzamine (Smith, Kline & French) was dissolved in a small volume of propylene glycol and diluted with water. Doses and routes of administration are indicated in the results section. Doses refer to free base forms of the drugs. The dose of clozapine used in this study (20 mg/kg) was selected as it is within the dose range studied in previous investigations (Bartholini *et al.*, 1972, 1973).

The marked hypothermia caused by clozapine administration was prevented by keeping clozapine-treated rats in a ventilated chamber at 32°C (Bartholini *et al.*, 1972).

Results

Administration of clozapine (20 mg/kg s.c.) every 2 h for 6 h resulted in a significant lowering of heart NA (Table 1), a lowering which was blocked by prior treatment with the ganglionic blocking drug, chlorisondamine. These results are qualitatively similar to the action of clozapine seen in brain and spinal cord and the effect of spinal transaction (Bartholini *et al.*, 1973).

This regimen of clozapine also lowered, but to a much greater extent (76%), the concentration of metaraminol (MA) in hearts of rats pretreated with the MA precursor. Pretreatment with chlorisondamine largely blocked MA release following clozapine. The greater effect of clozapine on MA than on NA is probably due to the fact that synthesis of NA tends to maintain the endogenous concentration of this amine while MA is not further synthesized. Since MA and NA responded in a qualitatively similar manner, further studies on the action of clozapine on the heart adrenergic neurone were carried out using MA measurement as the index of amine releasing action.

The experiments described above indicate that clozapine causes NA and MA release from heart by a nerve impulse-dependent mechanism. If clozapine were acting solely by virtue of its α-adrenoceptor blocking action so as to enhance reflexly adrenergic nerve traffic, then other α-blockers should mimic the action of clozapine. However, as shown in Table 1, neither phenoxybenzamine nor phentolamine caused a significant lowering of heart MA, suggesting that clozapine exerts an action in addition to that of α-blockade.

One possibility for a second action of clozapine would be inhibition of re-uptake of amine by the adrenergic neurone following release. However, according to Stille *et al.* (1971), clozapine has only a weak inhibitory action on the neuronal amine pump. Verification of this conclusion was obtained in experiments described in Table 2. In these experiments, the retention by the heart of MA given to rats was measured in the presence of a ganglionic blocking drug so as to eliminate the factor of impulse flow. The hearts of control rats accumulated considerable MA. Desipramine (desmethylimipramine), a potent inhibitor of the adrenergic neurone amine pump, markedly inhibited MA accumulation but clozapine had only a slight effect, indicating that clozapine has little effect on the amine uptake system.

Table 1 Action of clozapine and other α-adrenoceptor blocking agents on heart noradrenaline (NA) or metaraminol (MA) and effect of ganglionic blockade

	NA (µg/g ± s e)		*MA* (µg/g ± s.e.)	
Control	1 38 ± 0.06	(4)	0.49 ± 0.01	(11)
Clozapine	1.08 ± 0 09[a]	(7)	0.12 ± 0 01[c]	(10)
Phenoxybenzamine	—		0.43 ± 0 05[b,e]	(4)
Phehtolamine	—		0.50 ± 0.01[b,e]	(4)
Chlorisondamine	—		0 52 ± .05[d,e]	(7)
Clozapine + chlorisondamine	1.24 ± 0 07[b]	(8)	0 41 ± 0 01[b]	(6)

NA or MA were measured 6 h after treatment of rats with clozapine (20 mg/kg, s c every 2 h), phentolamine (20 mg/kg, s.c. every 3 h) or phenoxybenzamine (15 mg/kg i p) Chlorisondamine, when used was given (5 mg/kg, s.c) 6 h before killing animals (30 min before clozapine). Numbers in parentheses denote number of experiments.

(*a*) differs from control value ($P<0.025$); (*b*) no significant difference from control; (*c*) differs from control value ($P<0.001$); (*d*) differs from control ($P<0.05$); (*e*) differs from clozapine ($P<0.001$).

Despite these findings, we were concerned with the possibility that even a low degree of amine uptake blockade, when coupled with marked adrenergic impulse flow might be responsible for the clozapine-induced amine release. Experiments in which both phentolamine and desipramine were given to rats showed the expected finding that, over a 6 h period, a marked depletion of heart MA resulted, a depletion which was blocked by a ganglionic blocking agent, in this case, mecamylamine (Table 3).

Since relatively little MA was released in a 1 h period even by the combination of phentolamine and desipramine (Table 4), experiments were carried out at this short time to investigate whether clozapine would show an additional releasing action in the presence of the other drugs. As shown in Table 4, a single dose of clozapine caused a further lowering of heart MA, suggesting that the drug exerts an action directly on the adrenergic neurone, an action over and above that of enhanced impulse flow or possible uptake inhibition. Other antipsychotic drugs, haloperidol, chlorpromazine or thioridazine did not share this action of clozapine (Table 4). The β-adrenoceptor blocking drug, propranolol, did not facilitate release, nor did atropine (Table 4), suggesting that the direct neuronal action of clozapine was not associated with blockade of a presynaptic β-adrenoceptor or muscarinic site. Atropine, in fact, inhibited release of MA, possibly by inhibition of ganglionic transmission.

The experiments described thus far indicate that, in contrast to some other antipsychotic drugs and α-adrenoceptor blocking agents, clozapine has a direct facilatory action on amine release working in concert with enhanced nerve impulse flow. This action appears not to be associated with presynaptic α- or β-adrenoceptor sites or with a muscarinic site. One other possible mechanism for clozapine's action might be a

Table 2 Effect of desipramine and clozapine on the adrenergic neurone amine uptake system

		Heart MA (µg/g ± s.e.)	*Inhibition (%)*
Control	(8)	0.50 ± 0.02	–
Desipramine	(4)	0.12 ± 0.01*	76
Clozapine	(4)	0.42 ± 0.02†	16

Rats were given chlorisondamine (5 mg/kg, s.c.). Desipramine (3 mg/kg, s.c.) was given 30 min later. Clozapine (20 mg/kg, s.c.) was given 90 min before and 30 min after chlorisondamine. (–)-Metaraminol (MA) (50 µg/kg, i.v.) was given 1 h after chlorisondamine and rats were killed 1 h later. Numbers in parentheses denote numbers of experiments.
*Differs from control or clozapine ($P<0.001$); †Differs from control ($P<0.025$).

Table 3 Release of heart metaraminol (MA) by the combination of phentolamine and desipramine. Inhibition of release by ganglionic blockade

Treatment		*Heart MA (µg/g ± s.e.)*
Control	(8)	0.51 ± 0.03
Phentolamine	(8)	0.50 ± 0.01 NS
Desipramine	(4)	0.44 ± 0.03*
Phentolamine + desipramine	(4)	0.17 ± 0.03†
Phentolamine + desipramine + mecamylamine	(4)	0.47 ± 0.03

Rats were given desipramine (3 mg/kg s.c.) or phentolamine (20 mg/kg s.c.). When used in combination, phentolamine was given 30 min before desipramine. Mecamylamine, where used was given 30 min (5 mg/kg i.p.) before desipramine. Rats were killed 6 h after phentolamine. Numbers in parentheses denote numbers of experiments.
NS No significant difference from control, *Differs from control ($P<0.05$), †Differs from other values ($P<0.001$).

Table 4 The effect of clozapine and other agents on heart metaraminol (MA) when given to rats that had also received phentolamine and desipramine

Treatment		*Heart MA (µg/g ± s.e.)*
Control (Phentolamine + desipramine)	(20)	0.32 ± 0.01
Clozapine (20 mg/kg)	(12)	0.15 ± 0.02*
Haloperidol (2 mg/kg)	(4)	0.32 ± 0.03 NS
Chlorpromazine (20 mg/kg)	(11)	0.28 ± 0.02 NS
Thioridazine (20 mg/kg)	(11)	0.34 ± 0.03 NS
Propranolol (2 mg/kg)	(4)	0.37 ± 0.04 NS
Atropine (20 mg/kg)	(4)	0.47 ± 0.05†

Rats were given phentolamine (20 mg/kg s.c.) and desipramine (3 mg/kg s.c.). These rats served as controls. Other rats also received, at the same time, clozapine or one of the other agents listed. All agents were given subcutaneously. Rats were killed 1 h after drug treatment. Numbers in parentheses denote numbers of experiments.
*Differs from control ($P<0.001$), NS No significant difference from control, †Differs from control ($P<0.005$).

direct and selective effect on adrenergic storage granules so as to facilitate impulse-related release. Consistent with this possibility are previously described studies showing that reserpine-induced release of peripheral catecholamines is delayed by interruption of efferent adrenergic impulse flow (Holzbauer & Vogt, 1956; Brodie, Olin, Kuntzman & Shore, 1957; Hertting, Potter & Axelrod, 1962). This phenomenon was verified in the system described in the present studies. Thus, Table 5 shows that the release of MA from rat heart, when measured a short time after reserpine, was greatly inhibited by the presence of a ganglionic blocking drug.

Discussion

The present results show that administration of clozapine brings about the lowering of NA or MA concentrations in rat heart and that the effect is prevented by ganglionic blockade, indicating that the action requires neuronal impulse flow. This finding is in accord with the described effect of this drug on brain and spinal cord and inhibition of release by blockade of impulse flow, an observation which led to the suggestion that the depleting action of clozapine resides in its ability to block α-adrenoceptors, with a consequent feedback activation of NA neurones to such an extent that synthesis of the transmitter cannot keep pace with release (Bartholini *et al.*, 1972, 1973). However, it would seem that if this were the case, other α-receptor blocking drugs should share this action of clozapine, but neither phentolamine nor phenoxybenzamine significantly altered heart MA concentrations, unless given in combination with the amine uptake bocker, desipramine. It seems clear from the experiments described in the present study that clozapine has little or no effect on amine uptake mechanisms in the adrenergic neuronal membrane. Clozapine caused pronounced further lowering of MA, even in the presence of phentolamine and desipramine. This finding would seem to exclude the possibility that compensatory activation of neuronal firing by α-receptor blockade following clozapine administration could alone cause the marked lowering of heart amine levels, but that there must exist some additional action of clozapine.

Table 5 The effect of reserpine alone and in combination with phentolamine and chlorisondamine on heart metaraminol (MA)

Treatment		*Heart MA (μg/g ± s.e.)*
Control	(6)	0.62 ± 0 02
Reserpine	(8)	0 32 ± 0.01*
Reserpine + phentolamine	(9)	0.30 ± 0 02*
Reserpine + phentolamine + chlorisondamine	(9)	0 50 ± 0.03†

Rats were given reserpine (2 mg/kg s.c) Others were given reserpine and phentolamine (20 mg/kg i p) simultaneously. In other rats the combination was given 30 min after treatment with chlorisondamine (5 mg/kg s c). Rats were killed 1 h after reserpine. Numbers in parentheses denote numbers of experiments.

* Differs from control ($P < 0\ 001$), † Differs from reserpine or reserpine + phentolamine ($P < 0\ 001$).

There are now many reports of the existence of α- or dopamine receptors at the adrenergic nerve terminal which regulate impulse-induced NA release (See review by Langer, 1974). It is tempting to suggest that an action of clozapine may be the blockade of one of these receptors. However, our *in vivo* experiments gave no support to this possibility, as we were unable to demonstrate a clozapine-like action on heart MA release by the administration of other α-receptor or dopamine receptor blocking drugs.

Another possible site of action of clozapine could be at adrenergic amine storage granules to facilitate amine release. Although clozapine does not share reserpine's pronounced depleting action on 5-hydroxytryptamine and dopamine, it may have an incomplete reserpine-like action selectively on the adrenergic neurone. This possibility is strengthened by the demonstration that the depletion of heart MA by reserpine is greatly slowed by interruption of nerve traffic. Clozapine thus may exert a labilizing effect on adrenergic storage vesicles so as to facilitate amine release by the nerve impulse.

This investigation was supported by a grant (MH-05831) from the U.S. Public Health Service. The authors are grateful for the gift of clozapine from Sandoz Laboratories.

References

ANDÉN, N.-E. & STOCK, G. (1973). Effect of clozapine on the turnover of dopamine in the corpus striatum and in the limbic system. *J. Pharm. Pharmac.*, **25**, 346–348.

ANGST, J., BENTE, D., BERNER, P, HEIMANN, H, HELMCHEN, H. & HIPPIUS, H. (1971). Das klinische Wirkungsbild von Clozapin. *Pharmakopsychiat.*, **4**, 201–214.

ANGST, J., JAENICKE, U , PADRUTT, A. & SCHARFETTER, Ch. (1971). Ergebnisse eines Doppel-blindversuchs von Clozapin (8-Chlor-11-(4-methyl-1-piperazinyl)-5-Hdibenzo(b,e)(1,4)diazepin im Vergleich zu Levomepromazin. *Pharmakopsychiat.*, **4**, 192–200.

BARTHOLINI, G., HAEFELY, W., JALFRE, M., KELLER, H.H. & PLETSCHER, A. (1972). Effects of clozapine on cerebral catecholaminergic neurone systems. *Br. J. Pharmac.* **46**, 736–740.

BARTHOLINI, G., KELLER, H.H. & PLETSCHER, A. (1973). Effect of neuroleptics on endogenous norepinephrine in rat brain. *Neuropharmac.*, **12**, 751–756.

BRODIE, B.B., OLIN, J.S., KUNTZMAN, R.G & SHORE, P A. (1957). Possible interrelationship between release of brain norepinephrine and serotonin by reserpine. *Science*, **125**, 1293–1294.

CROUT, J.R., ALPERS, H.S., TATUM, E.L & SHORE, P.A. (1964). Release of metaraminol from the heart by sympathetic nerve stimulation. *Science*, **145**, 828–829.

HERTTING, G., POTTER, L.T. & AXELROD, J. (1962). Effect of decentralization and ganglionic blocking agents on the spontaneous release of H^3-norepinephrine. *J. Pharmac. exp Ther.*, **136**, 289–292.

HOLZBAUER, M. & VOGT, M. (1956). Depression by reserpine of the noradrenaline concentration in the hypothalamus of the cat. *J. Neurochem.*, **1**, 8–11.

LANGER, S Z. (1974). Presynaptic regulation of catecholamine release. *Biochem. Pharmac.*, **13**, 1793–1800.

NEFF, N.H & COSTA, E. (1966). The influence of monoamine oxidase inhibition on catecholamine synthesis. *Life Sci.*, **5**, 951–959.

SHORE, P.A & ALPERS, H.S. (1964). Fluorometric estimation of metaraminol and related compounds. *Life Sci.*, **3**, 551–554.

SHORE, P A, BUSFIELD, D. & ALPERS, H.S. (1964). Binding and release of metaraminol: mechanism of norepinephrine depletion by α-methyl-*m*-tyrosine and related agents. *J. Pharmac. exp. Ther.*, **146**, 194–199.

SNYDER, S.H, BANERJEE, S.P., YAMAMURA, H.I. & GREENBERG, D. (1974). Drugs, neurotransmitters, and schizophrenia. *Science*, **184**, 1243–1253.

STILLE, G. & HIPPIUS, A. (1971). Kritische Stellungsnahme zum Begriff der Neuroleptika (anhand von pharmakologischen und klinischen Befunden mit Clozapin). *Pharmakopsychiat.*, **4**, 182–191.

STILLE, G, LAUENER, H. & EICHENBERGER, E. (1971). The pharmacology of 8 chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e][1,4] diazepine (clozapine). *Il Farmaco*, **26**, 603–625.

(Received August 11, 1975.
Revised November 3, 1975.)

Br. J. Pharmac. (1976), 56, 285–292

RAT UTERUS RENIN-LIKE ACTIVITY: EFFECT OF STIMULI AND HORMONES

Y. GUTMAN & M. MAZUR-RUDER

Department of Pharmacology, The Hebrew University-Hadassah School of Medicine, Jerusalem, Israel

1 The characteristics of renin-like activity in rat uterus were studied. The optimum temperature was 50°C and optimum pH 4.0. Potassium (100 mM) enhanced this activity but sodium or calcium had no effect.

2 Uterine renin-like activity was unchanged 24 h after bilateral nephrectomy.

3 Renin-like activity in the uterus increased slowly from birth to 4 weeks of age and faster between the 4th and 8th week.

4 Ovariectomy caused a considerable fall in uterine renin-like activity.

5 The following factors, known to affect renin secretion in the kidney, i.e., hypovolaemia, sodium loading, adrenalectomy, administration of desoxycorticosterone acetate and injection of isoprenaline, had no effect on uterine renin-like activity.

6 Stilboestrol, an oestrogen, caused a significant increase of uterine renin-like activity. Progesterone and testosterone had no significant effect on this activity but blocked the increase caused by stilboestrol.

7 During pregnancy there was a small but significant fall of renin-like activity in the uterus in the first week and a continuous increase throughout the later period of pregnancy.

8 It is concluded that uterine renin-like activity is independent of kidney renin and does not respond to stimuli affecting kidney renin. Uterine renin activity is hormone-dependent and may be governed by the ratio, oestrogen:progesterone.

Introduction

Renin-like activity has been demonstrated in several tissues: submaxillary glands of mice (Cohen, Taylor, Murakami, Michelakis & Inagami, 1972) and rats (Gutman, Levy & Shorr, 1973), brain (Ganten, Minnich, Granger, Hayduk, Brecht, Barbeau, Boucher & Genest, 1971) and in human as well as in rabbit uterus (Gross, Schaechtelin, Ziegler & Berger, 1964; Geelhoed, Vander & Carlson, 1970). During pregnancy significant changes in the plasma renin-angiotensin system have been demonstrated (Skinner, Lumbers & Symonds, 1972). However, the possible correlation of uterine renin-like activity and plasma renin has not been clearly demonstrated. Furthermore, although several types of stimuli (changes in sodium balance, mineralocorticoids, β-adrenoceptor activation, hypovolaemia) have been shown to affect the release of renin from the kidney, the possible physiological stimuli which affect uterine renin have not been established.

We have characterized the properties of a renin-like activity in rat uterus and have studied the action of various possible stimuli on this activity. The results are described in this paper.

Methods

Female albino rats of the Hebrew University strain, weighing 150–210 g were used throughout. In some experiments, specified in the results section, immature female rats of different ages, were used. The rats were killed under ether anaesthesia by stretching of the neck and the uteri were immediately removed. Homogenates were made of samples consisting of 2–3 uteri.

Operations

All operations were carried out under ether anaesthesia.

Bilateral adrenalectomy was performed through a mid-line incision in the back. Sham-operated rats underwent the same procedure but the adrenals were not removed. Three days after the operation, the uteri were removed.

Bilateral nephrectomy was performed through a midline incision of the abdomen. The same procedure was carried out in the sham-operated animals, but the kidneys were left intact. The uteri were removed 24 h after operation.

For ovariectomy both ovaries were removed through two small incisions along the ventrolateral sides of the abdomen. The same procedure was carried out in sham-operated rats, except for removal of the ovaries. The uteri were taken out on the tenth post-operative day.

Treatment

1. Stilboestrol was injected intramuscularly, 0.5–0.7 mg/kg, on the eighth day after ovariectomy.
2. Progesterone was injected intramuscularly, 5–7 mg/kg, on the eighth day after ovariectomy.
3. Testosterone was injected intramuscularly, 6.3–8.3 mg/kg, on the eighth day after ovariectomy.
4. Dexamethasone was injected subcutaneously, 2–2.7 mg/kg, on the eighth day after ovariectomy.
5. Combined treatment (a) Stilboestrol and progesterone were administered simultaneously (0.5–0.7 mg stilboestrol and 5–7 mg progesterone/kg, both i.m.) on the eighth day after ovariectomy. (b) Stilboestrol, (0.5–0.7 mg/kg, i.m.) and dexamethasone, (2–2.7 mg/kg, s.c.,) were injected simultaneously on the eighth day after ovariectomy. (c) Testosterone, (6.3–8.3 mg/kg, i.m.) and stilboestrol, (5–7) mg/kg, i.m.) were injected simultaneously on the eighth day after ovariectomy.

Control ovariectomized rats were injected with an equivalent volume of oil. Following the various treatments described, the uteri were removed on the third day after drug injection.

6. Desoxycorticosterone acetate (DOCA) 5–7 mg/kg, was injected intramuscularly for three days. Control rats were injected daily with equal volumes of oil. The uteri were removed on the fourth day.
7. Isoprenaline (in 0.9% w/v NaCl solution (saline)) was administered subcutaneously, 1 mg/kg to rats, 2 h after bilateral nephrectomy. Control rats were nephrectomized and injected with saline. The uteri were removed 2 h after the injection.
8. Hypovolemia was induced according to the method of Stricker (1966), by subcutaneous injection of 4 ml/rat of a 20% solution of Carbowax in saline. Control rats were injected with an equivalent volume of saline. The uteri were removed after 4 hours.
9. Sodium loading. The animals were given 1.7% NaCl solution to drink for five days. Control rats drank tap water. The uteri were removed on the fifth day.

Removal of uteri for assay

The uteri were carefully separated from the ovaries and cut at the junction with the vagina. The uteri were then cleaned of fat and attached blood vessels, blotted on filter paper and weighed. The uteri of pregnant rats were separated also from foeti and placentae.

Homogenization

The uteri were placed in distilled water (w:v = 1:10) and were then subjected to three consecutive cycles of freezing and thawing. They were then homogenized in a Virtis Homogenizer, model 45, speed position-30, for 3–5 minutes. The homogenate was centrifuged at 10,000 *g* for 30 min, at 4°C. The supernatant (enzyme solution) was kept at −20°C until assayed.

Assay of renin-like activity in uterus

The enzyme solution (1 ml) was incubated with 1 ml renin-substrate (prepared from plasma of nephrectomized rats, according to Boucher, Menard & Genest, (1967)), 1 ml of resin (Dowex 50WX2, 100–200 mesh, to bind and protect angiotensin formed in the reaction), 1 ml citrate-phosphate buffer (pH 4.0) and 1 ml KCl 0.5 M (final concentration of K^+ in the reaction mixture was 0.1 M). Incubation was carried out at 50°C for 2 h in a thermostatic bath with constant shaking. The reaction was stopped by immediate freezing. The reaction conditions differed from this protocol in the initial experiments, when the optimal conditions for the enzyme activity were investigated. The reaction mixture was passed through Dowex 50WX2 resin columns, washed with water and eluted with diethylamine and NH_4OH, as previously described (Gutman *et al.*, 1973). The angiotensin in the eluate was assayed in nephrectomized, male rats injected with pentolinium, as described by Boucher *et al.* (1967). The amount of angiotensin in the sample was assessed by comparison with standard solutions of angiotensin II (Hypertensin, CIBA).

Drugs

The following drugs were used: Val^5-hypertensin-asp-βamide (Angiotensin II; CIBA-GEIGY Ltd, Basel), polyethylene glycol, mol. wt. 20,000 (Carbowax; Chemorad, Tel-Aviv), dexamethasone (ampoules of 400 mg/ml in saline; Ikapharm, Ramat-Gan, Israel), desoxycorticosterone acetate (DOCA, 5 mg/ml ampoules Organon, Holland), isoprenaline sulphate a gift from Teva, Jerusalem), pentolinium (Ansolysen; May & Baker), progesterone (Luteopur, ampoules of 50 mg/ml in oil; Teva, Jerusalem), stilboestrol dipropionate (dissolved in oil; Ward, Blenkinsop & Co.) and testosterone (Homosteron, ampoules containing 25 mg/2 ml oil; Teva, Jerusalem).

Results

Characterization of renin-like activity in rat uterus

Effect of pH Figure 1 shows the effect of pH on renin-like activity in the rat uterus. Optimal activity

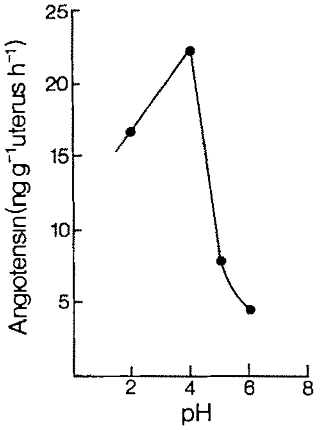


Figure 1 Effect of pH on renin-like activity of rat uterus Incubations carried out at 37°C, for 18 hours. Each point is the mean of 4 experiments.

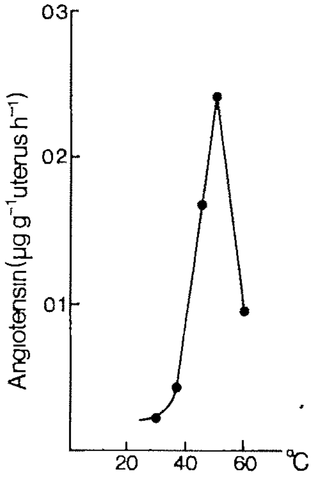


Figure 2 Effect of temperature on renin-like activity of rat uterus. Incubations carried out at pH 4, for 5 hours Each point is the mean of 3 experiments

was obtained at pH 4.0 with a sharp drop in activity at higher pH. Pre-incubation of the enzyme solution, without substrate, at pH ranging from 2 to 8, for 1 h, did not affect the activity of the enzyme on subsequent incubation with substrate at pH 4.0.

Effect of temperature Figure 2 shows the uterine renin-like activity at different temperatures. The optimal activity was obtained at 50°C with a sharp drop at lower and higher temperatures. Pre-incubation of the enzyme solution, without substrate, at temperatures of 4–50°C for 1 h caused no significant change of activity on subsequent incubation with substrate. Pre-incubation of the enzyme without substrate at 60°C for 1 hr caused inactivation of 65%.

The effect of various ions The effect of addition of various ions on renin-like activity in the rat uterus was next studied.

(a) Addition of Ca^{2+} (5–20 mM) or Na^+ (150 mM) had no significant effect on uterine renin-like activity.

(b) Addition of K^+ at final concentrations of 50–500 mM caused a substantial increase of the activity of the enzyme, with an optimum at 100 mM. At this concentration, activity was 2.25 times higher than in the absence of potassium.

Following these preliminary experiments, optimal assay conditions were set at 50°C, pH 4.0 and K^+ 100 mM.

Changes in uterine renin-like activity with age

Uterine renin-like activity was assayed in rats aged 1, 2, 4 and 8 weeks. Figure 3 shows that the renin-like activity rose slowly up to 4 weeks of age. A sharp elevation was noted between the fourth and eighth week.

The source and function of uterine renin-like activity

Bilateral nephrectomy To ascertain whether uterine renin-like activity originated from the kidney, bilateral nephrectomy was performed in female rats. Twenty four hours after nephrectomy, when plasma renin had completely disappeared, uterine renin-like activity showed no significant change, compared to sham operated rats, as shown in Table 1. This experiment shows that uterine renin-like activity is independent of kidney renin.

Hypovolaemia This is one of the major stimuli for release of renin from the kidney. To study further a possible correlation of kidney and uterine renin, hypovolaemia was induced in female rats by subcutaneous injection of the polymer polyethylene-glycol (Carbowax). No significant change in renin-like activity in the uterus was observed, compared to saline-injected rats, as shown in Table 1.

Na^+ loading Another stimulus which affects kidney renin, sodium loading, was given to female rats. No significant change in uterine renin-like activity was found, as seen in Table 1. Such treatment decreases kidney renin (Gross 1967, Vander 1970).

Effect of DOCA Mineralocorticoid administration causes a decrease of kidney and plasma renin (Pettinger, Marchell & Augusto, 1971). To

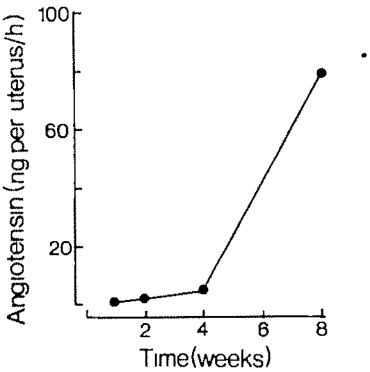


Figure 3 Effect of age on renin-like activity of rat uterus. Incubations carried out at 50°C, pH 4 for 2 hours. Each point is mean of two experiments

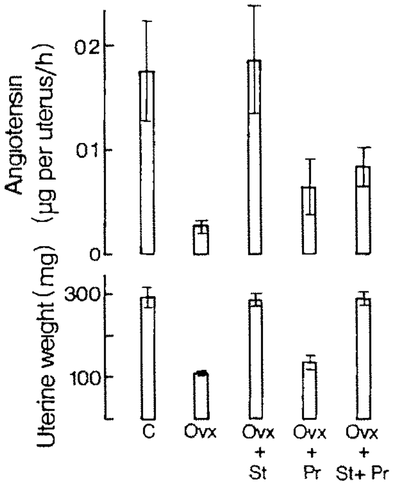


Figure 4 Effect of stilboestrol, progesterone and combined treatment on renin-like activity of rat uterus. Incubations carried out at 50°C, pH 4 for 2 hours. Vertical bars show s.e. mean. For renin-activity. C=control ($n=8$); Ovx=ovariectomy ($n=7$); St=stilboestrol ($n=7$); Pr=progesterone ($n=5$); St+Pr ($n=8$).

characterize further uterine renin-like activity, desoxycorticosterone acetate was injected intramuscularly into female rats. Table 1 shows that no significant change in uterine renin-like activity was observed.

Bilateral adrenalectomy Adrenalectomy causes a significant increase in kidney renin (Hasegawa, Nasjletti, Rice & Masson, 1973), but as seen in Table 1, there was no significant change in uterine renin-like activity compared to sham-operated rats.

Isoprenaline Activation of β-adrenoceptors causes release of renin from the kidney. Table 1 shows that no significant change in uterine renin followed injection of isoprenaline.

Effect of stilboestrol, progesterone and testosterone Figure 4 shows the effect of stilboestrol and progesterone, given separately or combined, to

Table 1 Effect of various treatments on uterine renin-like activity

	Renin-like activity (Angiotensin, ng per uterus/h) Control	Experimental	P
Nephrectomy	176±57 (6)	186±50 (8)	NS
Isoprenaline	148±45 (5)	155±20 (8)	NS
Adrenalectomy	104±20 (8)	109±12 (10)	NS
DOCA	124±18 (14)	148±66 (7)	NS
Na^+ loading	124±18 (14)	164±17 (8)	NS
Hypovolaemia	124±18 (14)	115±35 (9)	NS

Figures in parentheses indicate *n*. Nephrectomy—bilateral nephrectomy was performed in the experimental group and sham-operation in the control group, 24 h before removal of uteri. Isoprenaline—bilateral nephrectomy was performed 2 h before administration of saline (control) or isoprenaline, (1 mg/kg, s.c.), (experimental). Adrenalectomy—bilateral adrenalectomy was performed in the experimental group and sham-operation in the control group, 3 days prior to removal of uteri. DOCA—The mineralocorticoid was injected (1.0 mg/rat, i.m.) for three consecutive days; control rats were injected with the equivalent volume of oil. Uteri were removed on the fourth day. Na^+ loading—rats were given 1.7% NaCl to drink for 5 days, before removal of uteri, control rats were given tap water. Hypovolaemia—was induced by subcutaneous injection of 4 ml/rat of 20% Carbowax in saline, in the experimental group and of saline in the control group, 4 h before removal of uteri. Results are expressed as mean±s.e. mean, NS—difference between control and experimental group not significant.

ovariectomized rats. Uterine renin-like activity decreased significantly after ovariectomy as compared to sham-operated rats. When ovariectomized rats were treated with stilboestrol (oestrogen-like) there was a significant rise in renin-like activity compared to that of ovariectomized rats. Renin-like activity in uteri of ovariectomized rats treated with stilboestrol was not significantly different from that of control, sham-operated rats.

Ovariectomized rats treated with progesterone showed a small but significant elevation of uterine renin-like activity compared to ovariectomized rats injected with oil (figure 4). However, the activity was considerably lower than in control, sham-operated rats.

Ovariectomized rats treated with both stilboestrol and progesterone showed a significantly lower renin-like activity in the uterus than ovariectomized rats treated with stilboestrol alone. Thus, when both hormones were administered simultaneously their effect was not additive but antagonistic (Figure 4).

The results were similar when renin activity was compared per gram uterus. Thus, in the control group the activity was 601 ng angiotensin per g uterus/h; after ovariectomy the activity diminished to 224 ng g^{-1} h^{-1}; following stilboestrol administration to the ovariectomized rats the activity increased to 799 ng g^{-1} h^{-1}, after progesterone administration to ovariectomized rats-467 ng g^{-1} h^{-1}, while combined treatment of ovariectomized rats with stilboestrol and progesterone resulted in renin activity of only 351 ng g^{-1} h^{-1}, considerably below that of stilboestrol-treated rats.

In order to find whether the action of progesterone was non-specific, i.e. a function of its corticosteroid property, the effect of the corticosteroid dexamethasone was studied. Ovariectomized rats were given either stilboestrol or dexamethasone, or a combined treatment. Dexamethasone had no effect on uterine renin-like activity in ovariectomized rats or on the elevation of uterine renin caused by stilboestrol in ovariectomized rats, calculated as renin activity per uterus or per gram uterus (Table 2).

Testosterone administration reduced uterine renin-like activity in ovariectomized rats when calculated per gram uterus and reduced the effect of stilboestrol on uterine renin (decrease of 37.5 ± 7.2%) without a significant change in uterine weight (Table 3).

Changes in uterine renin-like activity during pregnancy

Dog and rabbit uterine renin have been reported to increase during pregnancy (Johnson, Ryan & Reyes-

Table 2 Effect of dexamethasone and stilboestrol on renin-like activity of rat uterus

Treatment	*Renin-like activity* *(Angiotensin, ng per uterus/h)*	*(Angiotensin ng g^{-1} h^{-1})*	*Uterine weight (mg)*
Ovariectomized	5.5	65.5	84
Ovariectomized + Stilb	68.0	246.4	276
Ovariectomized + Dexa	6 4	65 3	98
Ovariectomized + Stilb + Dexa	67 0	234 3	286

Stilb = stilboestrol (0 1 mg/rat, i.m.) Dexa = dexamethasone (0 4 mg/rat, s c) Each value is the mean of two experiments. The incubations with renin-substrate were carried out without the addition of K^+, therefore the activities were lower than in other experiments

Table 3 Effect of testosterone and stilboestrol on renin-like activity of uterus

Treatment	*Renin-like activity* *(Angiotensin, ng per uterus/h)*	*(Angiotensin ng g^{-1} h^{-1})*	*Uterine weight (mg)*
Ovariectomized	51 ± 10 (3)	526	97 ± 14 (9)
Ovariectomized + Stilb	198 ± 47 (3)	723	274 ± 8 (7)
Ovariectomized + Test	56 ± 14 (3)	357	157 ± 12 (9)
Ovariectomized + Stilb + Test	121 ± 38 (6)	465	260 ± 14 (18)

Figures in parentheses indicate *n*. Stilb = stilboestrol (0 1 mg/rat, i.m.) Test = testosterone (1 25 mg/rat, i m)
Results given as mean ± s.e. mean

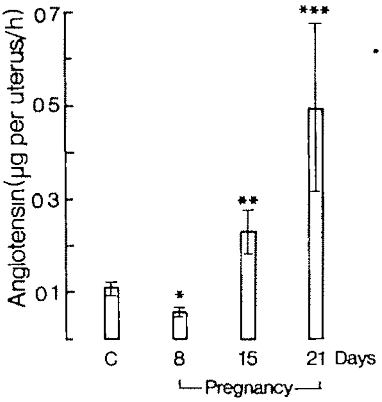


Figure 5 Effect of pregnancy on renin-like activity of rat uterus. Incubations carried out at 50°C, pH 4, for 2 hours. Vertical bars show s.e mean. Control, $n=11$, 8 days pregnancy, $n=5$; 15 days pregnancy, $n=6$; 21 days pregnancy, $n=3$.

Rodrigues, 1971). The effect of pregnancy in the rat is shown in Figure 5: there was a significant initial decrease in uterine renin-like activity (44%) at the beginning of pregnancy, and a steady rise later in pregnancy. Renin-like activity was also assayed in the placentae. The mean content of renin-like activity per placenta increased from 21 at 15 days of pregnancy, to 53 ng angiotensin released per placenta/h, on the last (21st) day of pregnancy.

Discussion

The presence of renin-like activity in extra-renal tissues has been reported in several species. Gross *et al.* (1964) described such renin-like activity in the placenta and uterus of the rabbit. Gutman *et al.* (1973) have identified such activity in the submaxillary gland of the rat, and Ganten *et al.* (1971) in the brain of the dog.

Recently, Carretero, Bujak & Houle (1971) showed that the various extra-renal renin-like enzymes catalyze the production of the same product, angiotensin. Gross *et al.* (1964) have shown that the final product of incubation of rabbit uterus homogenate is angiotensin and Hodary, Carretero & Hodgkinson (1969) demonstrated that angiotensin is formed upon incubation of dog uterine homogenate. Geelhoed *et al.* (1970) have shown the presence of renin-like enzyme in the human uterus. However, whereas the role of kidney renin in sodium balance is well established (Brown, Lever & Robertson, 1967), no functional significance of extra-renal renin has been clearly demonstrated.

One possibility is that extra-renal renin merely reflects adsorption of circulating renin from the blood into various tissues. This possibility has been eliminated in the case of the rat submaxillary gland (Gutman *et al.*, 1973) as well as for the utero-placental complex of the dog (Carretero, Polomski, Piwonska, Afsari & Hodgkinson, 1972) and the rabbit uterus (Gordon, Ferris & Mulrow, 1967). The experiments reported in the present paper corroborate these findings in the case of the renin-like enzyme of the rat uterus. Twenty four hours after bilateral nephrectomy of the female rat there was no reduction in the uterine renin-like activity (Table 1), whereas plasma renin disappears completely within a few hours. However, these findings do not refute the possibility that uterine renin-like stores do originate from the kidney but are released very slowly.

The uterine renin-like enzyme has been shown to be similar though not identical to kidney renin in both the dog (Carretero & Houle. 1970) and rabbit (Anderson, Herbert & Mulrow, 1968). The present paper has also shown that although the rat uterus renin-like enzyme is similar to kidney renin, its characteristics differ from those of kidney renin in the pH optimum and the enhancement of the reaction by K^+ but not by Na^+.

The effects of physiological stimuli which regulate kidney renin were studied on the uterine renin-like enzyme. Hypovolaemia had no effect on the uterine enzyme, whereas it is known to stimulate the release of kidney renin (Gross 1967; Vander 1970). Na^+ loading also showed no significant effect on the uterine renin-like activity, though it inhibits the release of kidney renin (Vander, 1970). Injection of a mineralocorticoid (DOCA) had no effect on the uterine renin-like activity, but it decreases plasma renin level (Pettinger *et al.*, 1971). Bilateral adrenalectomy had no effect on the uterine enzyme, whereas it stimulates the release of kidney renin, due to the enhanced sodium loss. These findings suggest that uterine renin is not affected by the same physiological stimuli that regulate kidney renin.

Several authors have studied the renin-angiotensin system in plasma during pregnancy. Thus, Talledo (1967) found differences in renin-angiotensin during normal and toxaemic pregnancies; Skinner *et al.* (1972) analysed changes in the renin system during pregnancy. Both of these studies, as well as others, have shown elevation of plasma renin activity during pregnancy. Various reports have also shown an increase of uterine renin during pregnancy: Ferris, Gordon & Mulrow (1967), and Johnson, *et al.* (1971) found an increase of renin in rabbit uterus. However, the possible relation of plasma renin and uterine-renin during pregnancy has not been demonstrated. Furthermore, in view of the independence of uterine-renin response to stimuli which affect plasma renin, such a relationship seems rather remote. Therefore, uterine renin-like enzyme may have a local function rather than play a role in systemic renin-angiotensin.

The ontogenesis of renin-like activity in the uterus follows sexual maturation. Thus, Figure 3 shows a slow rise from birth to the age of 4 weeks, and a sharp rise between the fourth and eighth week, when the female rat reaches puberty. This course could be reversed by ovariectomy. As seen in Figure 4 ovariectomy of mature rats caused a profound fall of uterine renin-like activity. The decrease of this activity in ovariectomized rats could be prevented completely by administration of an oestrogen, stilboestrol, while progesterone induced only a small and incomplete recovery of uterine renin-like activity after ovariectomy. (Figure 4.) In view of the qualitatively similar effects of progesterone and an oestrogen, it was rather surprising that combination of these treatments in ovariectomized rats caused a smaller elevation of renin-like activity than the oestrogen alone. Progesterone, therefore, seems to antagonize the effect of oestrogen on uterine renin-like activity. The increase of uterine weight caused by the oestrogen was not antagonized by progesterone.

In a recent communication, Hsueh, Peck & Clark (1975), demonstrated reduction of the amount of oestrogen-receptor in the uterus of immature rats by treatment with progesterone. This could be a possible mechanism for the phenomenon described in the present paper. However, in our experiment, carried out in mature female rats, progesterone did not reduce the uterine weight-gain induced by stilboestrol. A similar finding, inhibition of the increase of uterine renin induced by stilboestrol, followed injection of testosterone, without an effect on uterine weight (Table 3). One could have ascribed the effect of progesterone on uterine renin to corticosteroid-like effects, but dexamethasone, a potent glucocorticoid, had no effect on the action of oestrogen and, on uterine renin (Table 2).

The effects of oestrogen and, possibly, of progesterone were reflected also in the rat uterine-renin during pregnancy. Figure 5 shows that there was a gradual increase in uterine renin during pregnancy, except for a significant fall during the first week. If progesterone antagonizes the effect of oestrogen on uterine renin, then the ratio oestrogen: progesterone will be the effective parameter. It is interesting, therefore, that from the reports of Wiest (1970) and of Yoshinaga, Hawkins & Stocker (1969), this ratio was lower during the first 10–12 days of pregnancy than during the later periods of pregnancy. It is suggested that uterine renin may depend on the ratio oestrogen: progesterone.

Y.G. is an Established Investigator of the Chief Scientist, Israel Ministry of Health. The results described in the paper were presented at the 33rd Meeting of the Israel Physiological and Pharmacological Society, Haifa, June 1975.

References

ANDERSON, R.C., HERBERT, P.N. & MULROW, P.J. (1968). A comparison of the properties of renin obtained from the kidney and the uterus of the rabbit. *Amer. J. Physiol.*, **215**, 774–778.

BOUCHER, R., MENARD, J. & GENEST, J. (1967). A micromethod for measurement of renin in the plasma and kidney of rats. *Can. J. Physiol. Pharmac.*, **45**, 881–890.

BROWN, J.J., LEVER, A.F. & ROBERTSON, J.I.S. (1967). Renin and angiotensin in health and disease. *Schweiz. Med. Wochsch.*, **97**, 1679–1687.

CARRETERO, O.A. & HOULE, J.A. (1970). A comparison of renin obtained from pregnant uterus and kidney of the dog. *Amer. J. Physiol.*, **218**, 689–692.

CARRETERO, O.A., BUJAK, B. & HOULE, J.A. (1971). Renin isozymes of extra-renal origin. *Amer. J. Physiol.*, **220**, 1468–1472.

CARRETERO, O.A., POLOMSKI, C., PIWONSKA, A., AFSARI, A & HODGKINSON, C.F. (1972). Renin release and the utero-placental complex *Amer. J. Physiol.*, **223**, 561–564

COHEN, S., TAYLOR, J. MURAKAMI, J., MICHELAKIS, A. & INAGAMI, T. (1972). Isolation and characterisation of renin-like enzymes from mouse submaxillary glands. *Biochemistry*, **11**, 4286–4292.

FERRIS, T.F., GORDEN, P. & MULROW, P.J. (1967). Rabbit uterus as a source of renin. *Amer. J. Physiol.*, **212**, 698–702.

GANTEN, D., MINNICH, J.L., GRANGER, D., HAYDUK, K., BRECHT, N.M., BARBEAU, A., BOUCHER, R. & GENEST, J. (1971). Angiotensin forming enzyme in brain tissue. *Science*, **173**, 64–65.

GEELHOED, G.W., VANDER, A.J. & CARLSON, J. (1970). Renin activity in the human uterus. *Proc. Soc. exp. Biol. Med.*, **133**, 479–484.

GORDON, P., FERRIS, T.F. & MULROW, P.J. (1967). Rabbit uterus as a possible site of renin synthesis. *Amer. J. Physiol.*, **212**, 703–706.

GROSS, F. (1967). The regulation of aldosterone secretion by the renin-angiotensin system under various conditions. *Acta. Endocrinol., Suppl.* **124**, 41–64.

GROSS, F., SCHAECHTELIN, G., ZIEGLER, M. & BERGER, M. (1964). A renin-like substance in the placenta and uterus of the rabbit. *Lancet*, **i**, 914–916.

GUTMAN, Y., LEVY, M. & SHORR, J. (1973). Renin-like activity of the rat submaxillary gland: characterization and the effect of several drugs and stimuli. *Br. J. Pharmac.*, **47**, 59–68.

HASEGAWA, H., NASJLETTI, A., RICE, K. & MASSON, G. (1973). Role of pituitary and adrenals in the regulation of plasma angiotensinogen. *Amer. J. Physiol.*, **225**, 1–6.

HODARY, A.A., CARRETERO, O.A. & HODGKINSON, C.P.

Br. J. Pharmac. (1976), **56**, 293–299

A METHOD FOR RECORDING SMOOTH MUSCLE AND VASCULAR RESPONSES OF THE BLOOD-PERFUSED DOG TRACHEA *In situ*

N. HIMORI & N. TAIRA
Department of Pharmacology and Experimental Therapeutics, Tohoku University School of Medicine, Sendai 980, Japan

1 A method is described for measuring responses of dog tracheal musculature and vasculature *in situ*.

2 The upper two thirds of the trachea was perfused with blood through both cranial thyroid arteries at a constant pressure. The blood flow through the arteries was measured with an electromagnetic flowmeter. The response of the tracheal musculature was measured as a change in pressure in a water-filled cuff inserted into the trachea *via* the mouth. Drugs were injected close-arterially.

3 Acetylcholine produced dose-dependent increases in blood flow rate (vasodilatation) and in tracheal intraluminal pressure (tracheal constriction). These responses were antagonized by atropine.

4 Isoprenaline produced vasodilatation which was blocked by propranolol. Adrenaline and noradrenaline caused vasoconstriction which was blocked by phentolamine.

5 All three catecholamines produced a decrease in tracheal intraluminal pressure (tracheal dilatation). The tracheal dilatation in response to adrenaline and noradrenaline was converted to constriction by propranolol. The tracheal constriction thus unmasked was abolished specifically by phentolamine.

6 From these results it is concluded that the tracheal musculature and vasculature contain muscarinic receptors, and excitatory α- and inhibitory β-adrenoceptors. In the tracheal musculature β-adrenoceptors predominate over α-adrenoceptors; the reverse is true in the tracheal vasculature.

Introduction

In the past decade many studies have been made of the physiological and pharmacological properties of the tracheal smooth muscle. Mammalian species used in these studies include the calf (Ariëns, 1967), the cat (Akçasu, 1959; Maengwyn-Davies, 1968; Fleisch, Maling & Brodie, 1970), the guinea-pig (Castillo & De Beer, 1947; Akçasu, 1959; Jamieson, 1962; Foster, 1966; Takagi, Osada, Takayanagi & Taga, 1967; Danko, Tozzi & Roth, 1968; Kasses, Beinfield, Seifter & Seifter, 1968; Chahl & O'Donnell, 1969; Everitt & Cairncross, 1969; Guirgis, 1969; Lynn James, 1969; Fleisch *et al.*, 1970; Rikimaru & Sudoh, 1971; Chahl, 1972; Coleman & Levy, 1974; Kirkpatrick, 1975), the rabbit (Akçasu, 1959; Fleisch *et al.*, 1970) and the rat (Akçasu, 1959; Fleisch *et al.*, 1970). However, most of the studies have been on isolated tracheal preparations bathed in physiological salt solutions. Only one study (Lynn James, 1969) used the *in vivo* tracheal preparation of the guinea-pig to assess drug action on the tracheal musculature. In view of this, it was felt that *in situ* recording of tone of the tracheal musculature with its nerve and blood supply intact would be of value and might reveal some features of the tracheal musculature which have previously escaped notice in *in vitro* experiments. Thus, in the present experiments we attempted to perfuse the tracheal vasculature with blood through the arteries and to record tracheal muscle tone in the dog. This method enabled us to inject drugs selectively into the tracheal vasculature and to observe simultaneously vascular and muscular responses of the trachea. The use of the method is illustrated with experiments on the interactions between cholinoceptor agonists and antagonists and adrenoceptor agonists and antagonists.

Part of this work was briefly reported at the 48th general meeting of the pharmacological society of Japan (Himori & Taira, 1975).

Methods

Experiments were performed on 38 adult mongrel dogs of either sex, weighing 12.5–18.0 kg. Anaesthesia was induced by a single intravenous injection of pentobarbitone sodium 30 mg/kg and

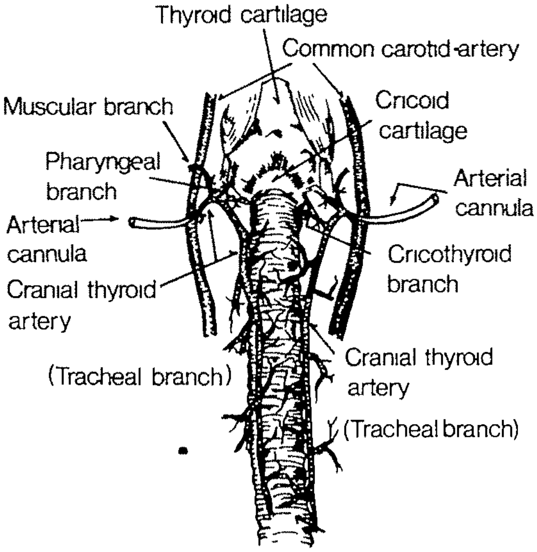


Figure 1 Arterial supply of the trachea of the dog. Arterial cannulae are inserted into the cranial thyroid arteries on both sides.

maintained by intravenous infusion of the same anaesthetic at a rate of 5 mg $kg^{-1}h^{-1}$.

The upper cervical region was incised in the midline, and the left and right cranial thyroid arteries, the main arteries supplying the upper portion of the trachea, and their branches were exposed and carefully dissected free. Muscular, pharyngeal and cricothyroid branches of the cranial thyroid arteries were all ligated, and branches supplying the trachea were left intact. Two arms of a Y-shape cannula were inserted into the left and right cranial thyroid arteries, as shown in Figure 1. Arterial blood from the right femoral artery was conducted to the Y-shape cannula *via* a peristaltic pump (Harvard Apparatus, Model 1215). Constant pressure perfusion was achieved by shunting a portion of blood through a Starling pneumatic resistance to the right femoral vein. The perfusion pressure was set at a value slightly lower than the mean systemic blood pressure at the beginning of perfusion and kept constant throughout the experiment. The perfusion pressure was monitored from a side arm of the perfusion circuit and the systemic blood pressure was measured from the femoral artery by pressure transducers (Nihon Kohden, MPU-0.5). The rate of blood flow through

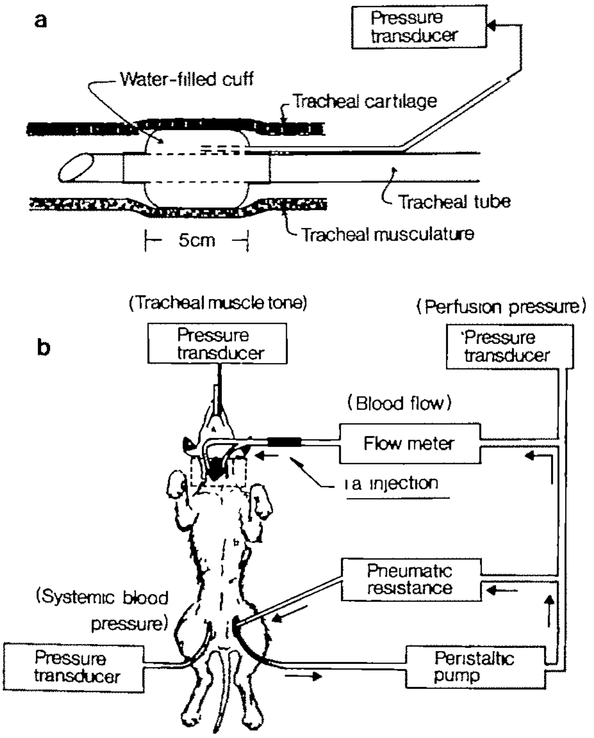


Figure 2 (a) Schematic illustration of the tracheal tube with a water-filled cuff attached for measurement of the tracheal intraluminal pressure. (b) Diagram of circuit for constant pressure perfusion of the tracheal vascular bed through the cranial thyroid arteries with blood from the femoral artery.

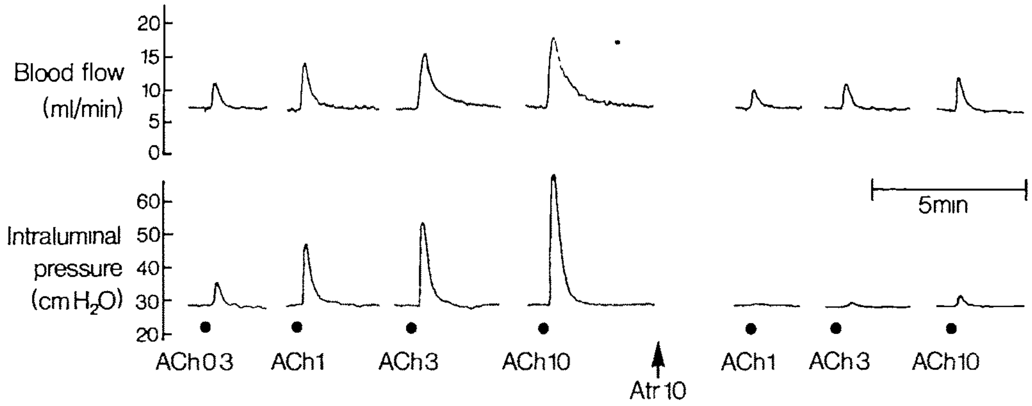


Figure 3 Responses of the tracheal vascular bed (blood flow rate) and musculature (intraluminal pressure) to intra-arterial acetylcholine (ACh) and antagonism by intra-arterial atropine (Atr) All doses in µg

the arteries was measured with an electromagnetic flowmeter (Nihon Kohden, MF-46-3) situated just proximal to the Y-shape cannula. Just before the start of perfusion the animal was given intravenous heparin sodium, 500 units/kg, and 100 units/kg were added intravenously at hourly intervals. Responses of the tracheal smooth muscle were measured as changes in the intraluminal pressure of a water-filled cuff (5 cm in length) attached to a tracheal tube, which was introduced into the trachea *via* the mouth (Figure 2a). The water-filled cuff was connected to a pressure transducer (Nihon Kohden, MPU-0.1) through polyethylene tubing (Intramedic PE 100). The water-filled cuff was located from 3 to 8 cm caudal to the larynx. The volume of water in the cuff was adjusted initially to give resting intraluminal pressures of 25–35 cmH_2O. This pressure range was found to be suitable for the observation of both dilatation and constriction of the trachea. Recordings were made on an ink-writing rectigraph (San-ei Instrument, Rectiholiz 8s). The experimental set-up is illustrated schematically in Figure 2b.

In 16 dogs at the conclusion of experiment, 0.4% w/v indigo carmine in 0.9% w/v NaCl solution (saline) was injected intra-arterially and the area perfused was checked. The upper two thirds of the trachea from the larynx was found to be clearly stained by the dye. In addition, the ventral upper third of the oesophagus was also slightly stained.

Drugs used in this study were acetylcholine chloride (Daiichi), atropine sulphate (Merck), (—)-adrenaline (Merck), (—)-noradrenaline (Fluka), (—)-isoprenaline hydrochloride (Nikken Kagaku), (±)-propranolol hydrochloride (ICI), phentolamine methanesulphonate (Ciba), 5-hydroxytryptamine creatinine sulphate (Wako) and methysergide tartrate (Sandoz). Adrenaline and noradrenaline were dissolved in 0.01 N HCl. All other drugs were dissolved in saline. All drug solutions were diluted with saline to the desired concentrations. Drug solutions in a volume of 30 µl (in 4 s) or 100 µl (in 15 s) were injected by the use of microsyringes into the rubber tubing just proximal to the Y-shape cannula.

Doses of acetylcholine, adrenaline, noradrenaline and 5-hydroxytryptamine refer to their bases and of atropine, propranolol, phentolamine and methysergide to their salts.

Values in the text are arithmetic means ±s.e. (unless otherwise stated). The statistical significance of the differences between mean values was analysed with Student's t-test and expressed as P values.

Results

Basal values of main parameters under resting conditions

The mean systemic blood pressure was 134.9 ± 3.7 mmHg ($n = 38$). The retrograde pressure measured by clamping the tubing just proximal to the side arm to the pressure transducer was 72.8 ± 2.4 mmHg ($n = 28$). The retrograde flow from the arterial cannula disconnected from the tubing was 4.1 ± 0.2 ml/min ($n = 28$). This was measured by the timed collection of blood in a graduated cylinder. The blood flow rate at a constant perfusion pressure of about 130 mmHg (127.5 ± 6.2 (s.d.) mmHg ($n = 38$)) was 9.5 ± 0.5 ml/min ($n = 38$). The preparation remained in good condition for 8 h or more.

Effects of acetylcholine on tracheal blood flow rate and smooth muscle tone

Single intra-arterial injections of acetylcholine (0.3–10 µg) produced dose-dependent increases in blood flow rate, *viz.*, vasodilatation and in intraluminal

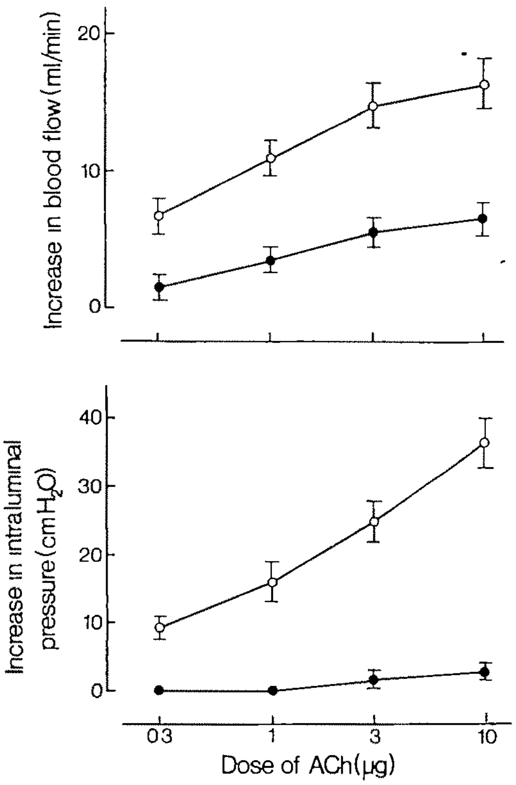


Figure 4 Dose-response curves for increase in rate of blood flow through and increase in intraluminal pressure of the trachea to intra-arterial acetylcholine before (○) and after (●) atropine (10 μg, i.a). Each point represents the mean of 14 observations on 14 animals Vertical bars show s e mean.

pressure, *viz.*, tracheal constriction. Figure 3 is typical of such experiments and Figure 4 shows dose-response curves for peak increases in the blood flow rate and intraluminal pressure. In some preparations, the tracheal constriction caused by acetylcholine was followed by tracheal dilatation. Intra-arterial injections of these doses of acetylcholine caused no systemic effect.

The vasodilatation and tracheal constriction caused by acetylcholine were antagonized by atropine, 10 μg intra-arterially ($P<0.01$) (Figures 3 and 4), the effect of atropine lasting for about 40 minutes. The tracheal dilatation following the constriction in response to acetylcholine observed in some preparations was also blocked by atropine.

Effects of (−)-isoprenaline, (−)-adrenaline and (−)-noradrenaline on tracheal blood flow rate and smooth muscle tone

Single injections of isoprenaline (0.03–1 μg, i.a.) caused dose-dependent increases in blood flow (vasodilatation) and decreases in intraluminal pressure (tracheal dilatation) (Figures 5 and 6). Adrenaline (0.1–1 μg, i.a.) and noradrenaline (0.3–3 μg, i.a.) produced vasoconstriction but like isoprenaline these two catecholamines caused tracheal dilatation in a dose-dependent manner (Figures 5 and 6). In only one of 13 preparations, including those used in preliminary experiments, 1 and 3 μg of noradrenaline increased intraluminal pressure (tracheal constriction) by about 12 and 20 cmH$_2$O, respectively. Relative potencies determined on the basis of doses producing a 10 cmH$_2$O decrease in the intraluminal pressure were in descending order: isoprenaline > adrenaline > noradrenaline, being approximately 1 : 1/3 : 1/12 (Figure 6). Adrenaline was more potent than noradrenaline in producing vasoconstriction ($P<0.05$) (Figure 6). Intra-arterial injections of these doses of the three catecholamines had no systemic effect.

Modification by (±)-propranolol and phentolamine of the effects of catecholamines

A single injection of propranolol (60 μg, i.a.) increased transiently (0.5–1 min) the blood flow rate by about 4.5 ml/min but had no effect on the tracheal intraluminal pressure. This dose of propranolol blocked the vasodilatation and tracheal dilatation caused by isoprenaline ($P<0.01$) and converted the tracheal dilatation in response to adrenaline and noradrenaline to constriction (Figures 5 and 6). In the presence of propranolol, adrenaline was 2–3 times as potent as noradrenaline in producing tracheal constriction ($P<0.05$). The tracheal constriction in response to adrenaline and noradrenaline after an intra-arterial injection of propranolol developed slowly in comparison with that to acetylcholine, as shown in Figure 5 (cf. Figure 3). The vasoconstriction caused by adrenaline and noradrenaline was not significantly different before and after propranolol ($P>0.6$) (Figure 6). The blocking effect of 60 μg of propranolol on the tracheal dilatation caused by isoprenaline, adrenaline and noradrenaline lasted for 30–40 minutes. This dose of propranolol did not modify the tracheal constriction caused by 10 μg of acetylcholine.

The tracheal constriction in response to adrenaline and noradrenaline in the presence of propranolol was almost abolished by a single intra-arterial injection of 200 μg of phentolamine ($P<0.01$), as shown in Figures 5 and 6. The vasoconstriction in response to adrenaline and noradrenaline was also antagonized by phentolamine ($P<0.05$) (Figures 5 and 6). The antagonism by phentolamine lasted for 50–60 minutes.

The tracheal constriction caused by adrenaline and noradrenaline after propranolol was not modified by either atropine (10 μg) or methysergide (3 μg); these doses abolished the tracheal constriction caused by

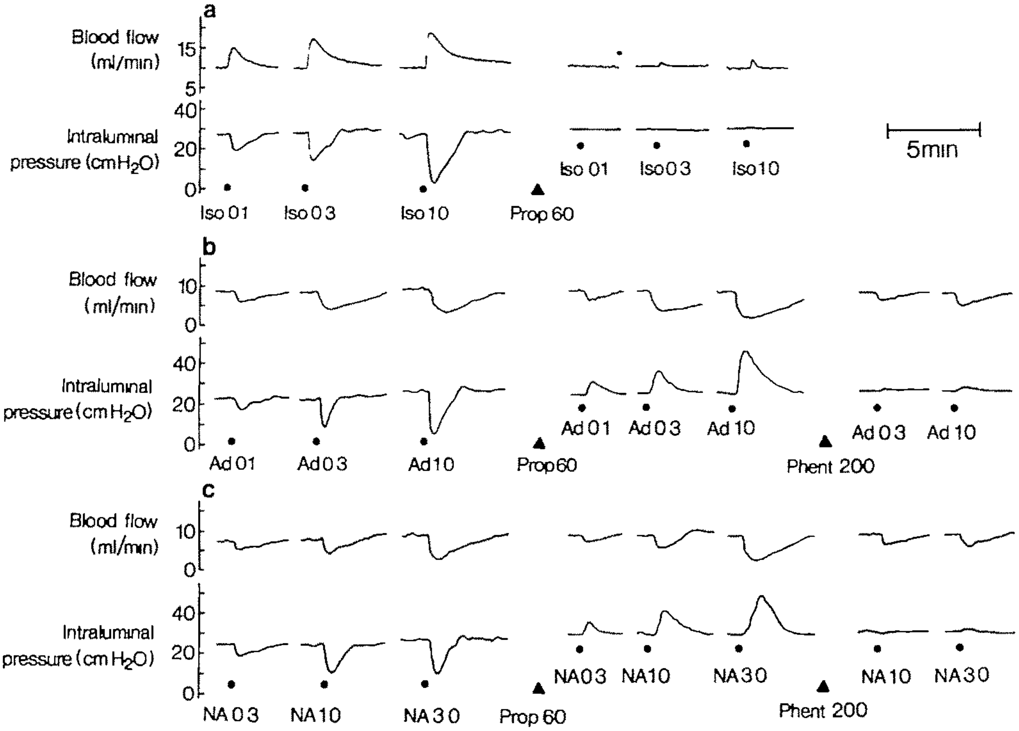


Figure 5 Responses of the tracheal vascular bed (blood flow rate) and musculature (intraluminal pressure) to (a) isoprenaline (Iso), (b) adrenaline (Ad) and (c) noradrenaline (NA), and modification by consecutive intra-arterial injections of propranolol (Prop) and phentolamine (Phent). All doses in μg

acetylcholine (3 μg) and 5-hydroxytryptamine (3 μg), respectively.

Discussion

In the present experiments the trachea of the dog was perfused *in situ* with arterial blood through the cannulated cranial thyroid arteries after all the branches except those supplying the trachea had been ligated. However, the upper portion of the oesophagus is also supplied by arterial branches which supply the upper trachea (Miller, 1964). Thus, the question arises whether a blood flow response as observed in the present experiments represents the perfused tracheal vascular bed alone. Indigo-carmine injected intra-arterially during the perfusion stained the upper two thirds of the trachea and to a lesser degree the ventral half of the upper third of the oesophagus, demonstrating that the vascular response as recorded is mainly that of the tracheal vascular bed. Therefore, the arterially blood-perfused tracheal preparation of the dog is suitable for pharmacological analysis of drug action on the tracheal musculature and vasculature *in situ*.

Acetylcholine caused vasodilatation and tracheal constriction and these effects were antagonized by atropine, indicating that they were mediated *via* stimulation of muscarinic receptors.

Isoprenaline, adrenaline and noradrenaline produced relaxation of the tracheal smooth muscle and the order of their relative potencies are consistent with that of the β-adrenoceptor stimulant activity of the three catecholamines determined on the tracheal smooth muscle of the calf (Ariëns, 1967) and guinea-pig (Guirgis, 1969; Chahl, 1972). The tracheal relaxations in response to the three catecholamines were blocked by propranolol. This indicates that the relaxation of the tracheal smooth muscle of the dog is mediated through β-adrenoceptors as demonstrated in the guinea-pig (Foster, 1966; Takagi *et al.*, 1967; Everitt & Cairncross, 1969; Guirgis, 1969; Chahl & O'Donnell, 1971; Chahl, 1972). In the presence of propranolol, adrenaline and noradrenaline but not isoprenaline caused tracheal constriction. Adrenaline was more potent than noradrenaline. Thus, the relative potencies of the two catecholamines coincide with those found for α-adrenoceptor stimulant activity in the vas deferens of the rat (Ariëns, 1967) and in the isolated tracheal chain of the guinea-pig in the

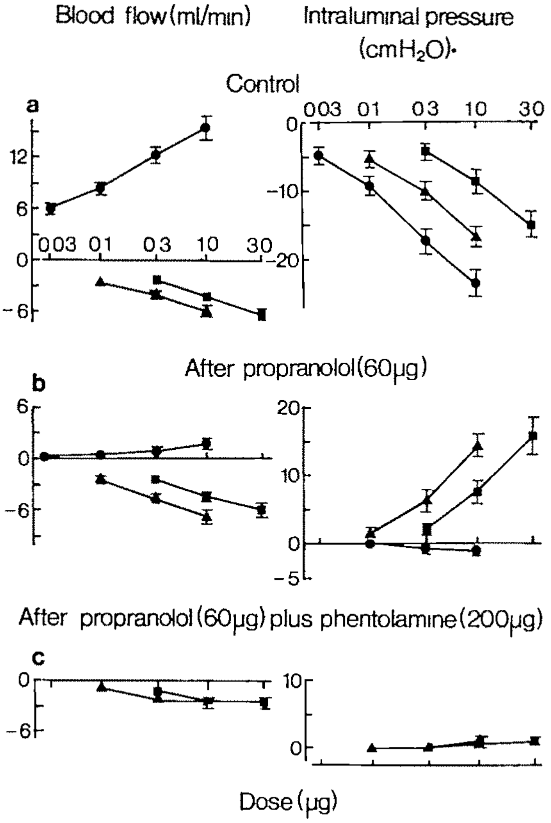


Figure 6 Dose-response curves for changes in rate of blood flow through (left side of figure) and intraluminal pressure (right side of figure) of the dog trachea to intra-arterial isoprenaline (●), adrenaline (▲) and noradrenaline (■), (a) before and (b) after a single injection of propranolol (60 µg, i.a.) and (c) after a further single injection of phentolamine (200 µg, i.a.). Each point represents the mean of 5–7 observations on 5–7 animals. Vertical bars show s.e. mean.

presence of propranolol (Everitt & Cairncross, 1969). The relative potencies also satisfy the criteria of Ahlquist (1966) which characterize α-adrenoceptors. The tracheal constriction was antagonized specifically by phentolamine. These results clearly indicate that the constrictor action of the two catecholamines on the dog trachea was mediated through excitatory α-adrenoceptors. The existence of the excitatory α-adrenoceptors in the tracheal smooth muscle has been demonstrated in the guinea-pig isolated tracheal preparations (Takagi *et al.*, 1967; Kasses *et al.*, 1968; Everitt & Cairncross, 1969; Fleisch *et al.*, 1970), although some investigators (Foster, 1966; Danko *et al.*, 1968; Rikimaru & Sudoh, 1971) failed to demonstrate it in the isolated guinea-pig trachea.

Isoprenaline caused tracheal vasodilatation, whereas adrenaline and noradrenaline caused tracheal vasoconstriction. The vasodilatation in response to isoprenaline was blocked specifically by propranolol, and the vasoconstriction in response to adrenaline and noradrenaline was antagonized specifically by phentolamine. This indicates that the vasodilatation was mediated through β-adrenoceptors whereas the vasoconstriction was mediated through α-adrenoceptors in the vasculature.

These results taken together indicate that inhibitory β-adrenoceptors predominate over excitatory α-adrenoceptors in the tracheal musculature and *vice-versa* in the tracheal vasculature of the dog.

This study was supported by grants from Research Laboratories, Teikoku Hormone, Kawasaki and from Research Laboratories, Otsuka Pharmaceutical Co., Tokushima, Japan.

References

AHLQUIST, R.P. (1966). The adrenergic receptor. *J. pharm. Sci.*, **55**, 359–367.

AKÇASU, A. (1959). The physiologic and pharmacologic characteristics of the tracheal muscle. *Arch. int. Pharmacodyn.*, **122**, 201–207.

ARIËNS, E.J. (1967). The structure-activity relationships of beta adrenergic drugs and beta adrenergic blocking drugs. *Ann. N.Y. Acad. Sci.*, **139**, 606–631.

CASTILLO, J.C. & DE BEER, E.J (1947). I. A preparation for the study of antispasmodics with particular reference to bronchodilator drugs. *J. Pharmac. exp. Ther.*, **90**, 104–109.

CHAHL, L.A. (1972). A structure-activity study of sympathomimetic amines on the beta-adrenoceptors of guinea-pig trachea. *Br. J. Pharmac.*, **45**, 510–518.

CHAHL, L A. & O'DONNELL, S.R. (1969). Actions of phenylephrine on beta-adrenoceptors in guinea-pig trachea. *Br. J. Pharmac.*, **37**, 41–51.

CHAHL, L A. & O'DONNELL, S.R. (1971). The effects on some receptors of guinea pig trachea of a series of sympathomimetic amines. *Eur. J. Pharmac.*, **16**, 201–208.

COLEMAN, R.A & LEVY, G.P. (1974). A non-adrenergic inhibitory nervous pathway in guinea-pig trachea. *Br. J. Pharmac.*, **52**, 167–174.

DANKO, G., TOZZI, S. & ROTH, F.E. (1968). Determination of the adrenergic receptor mechanism of the isolated guinea pig trachea utilizing a new technique. *Fed. Proc.*, **27**, 351.

EVERITT, B.J. & CAIRNCROSS, K.D (1969). Adrenergic receptors in the guinea-pig trachea. *J. Pharm. Pharmac.*, **21**, 97–102.

FLEISCH, J.H., MALING, H.M. & BRODIE, B.B. (1970). Evidence for existence of alpha-adrenergic receptors in the mammalian trachea. *Am. J. Physiol.*, **218**, 596–599.

FOSTER, R.W. (1966). The nature of the adrenergic receptors of the trachea of the guinea-pig. *J. Pharm. Pharmac.*, **18**, 1–12.

GUIRGIS, H.M. (1969). Potentiation of the effects of catecholamines by α- and their antagonism by β-adrenergic blocking agents on the guinea-pig isolated trachea *in vitro* and on the guinea-pig lungs *in vivo*. *Arch int. Pharmacodyn.*, **178**, 31–42.

HIMORI, N. & TAIRA, N. (1975). *In situ* perfusion of the tracheal vascular bed of the dog—A new method for tracheal pharmacology. *Jap. J. Pharmac. (Suppl.)*, **25**, (in press).

JAMIESON, D. (1962). A method for the quantitative estimation of drugs on the isolated intact trachea. *Br. J. Pharmac.*, **19**, 286–294.

KASSES, K, BEINFIELD, W.H., SEIFTER, E & SEIFTER, J. (1968). Alpha receptors in isolated guinea pig tracheal chain. *Pharmacologist*, **10**, 206.

KIRKPATRICK, C.T. (1975). Excitation and contraction in bovine tracheal smooth muscle. *J. Physiol., Lond.*, **244**, 263–281.

LYNN JAMES, G.W. (1969). The use of the *in vivo* trachea preparation of the guinea-pig to assess drug action on lung. *J. Pharm. Pharmac.*, **21**, 379–386.

MAENGWYN-DAVIES, G.D. (1968). The dual mode of action of histamine in the cat isolated tracheal chain. *J. Pharm. Pharmac.*, **20**, 572–573.

MILLER, M.E. (1964). *Anatomy of the dog*. Philadelphia: Saunders.

RIKIMARU, A. & SUDOH, M. (1971). Innervation of the smooth muscle of the guinea-pig trachea. *Jap. J. Smooth Muscle Res.*, **7**, 35–44.

TAKAGI, K., OSADA, E., TAKAYANAGI, I. & TAGA, F. (1967). Adrenergic receptors on some organs. *Arch. int. Pharmacodyn.*, **168**, 212–219.

(Received August 29, 1975.
Revised November 7, 1975.)

Br. J. Pharmac. (1976), **56**, 301–305

IMPAIRED ABSORPTION OF CHLORPROMAZINE IN RATS GIVEN TRIHEXYPHENIDYL

LEONOR RIVERA-CALIMLIM
Department of Pharmacology and Toxicology, University of Rochester,
School of Medicine and Dentistry, Rochester, New York 14642, U.S.A.

1 The absorption and tissue distribution of orally administered [^{14}C]-chlorpromazine (CPZ) were compared in trihexyphenidyl (THP; Artane)-treated and control rats.

2 Total radioactivity (CPZ) in the plasma and brain of rats treated with THP was significantly lower whereas total radioactivity in the stomach was significantly higher than in rats not previously treated with THP.

3 Gastric emptying in rats treated with THP was significantly delayed as measured by gastric clearance of a marker, [^{14}C]-polyethylene glycol.

4 Transport of [^{14}C]-CPZ in everted sacs was not affected by treatment with THP.

5 Metabolism of [^{14}C]-CPZ by liver homogenates was not affected by treatment with THP.

6 The relationship of delayed gastric emptying in THP-treated rats and their lower plasma and brain levels of [^{14}C]-CPZ after oral administration is discussed.

Introduction

Trihexyphenidyl (THP; Artane), a parasympatholytic drug, has been used clinically in the symptomatic treatment of Parkinson's disease since 1949. Because of its antiparkinsonian activity, it has been used with neuroleptic drugs routinely or prophylactically in psychiatry to prevent the occurrence of extrapyramidal symptoms. Observations by Singh and colleagues (Singh & Smith, 1973; Singh & Kay, 1974; Singh & Kay, 1975) indicated reversal of the therapeutic effects achieved with neuroleptics when parasympatholytics were added to the therapeutic regimen. Concurrently, our double-blind studies in psychiatric patients (Rivera-Calimlim, Castañeda & Lasagna, 1973; Rivera-Calimlim, Nasrallah, Strauss & Lasagna, 1975) showed that trihexyphenidyl consistently lowers plasma levels of chlorpromazine (CPZ). The present study was intended to reproduce this trihexyphenidyl-CPZ interaction in rats and to investigate the site of this interaction.

Methods

Chronic treatment

Groups of eight male Sprague-Dawley rats (160–180 g), housed in individual cages and maintained on regular laboratory diet and water, were treated daily with trihexyphenidyl, administered orally, for two weeks. Trihexyphenidyl, (THP; Artane, Lederle Laboratories, Pearl River, New York) as a dose of 50 mg/kg was dissolved in water and given orally in a 1 ml solution once a day through a metal gastric tube. Matched controls received an equivalent volume of water.

At the end of the pretreatment period, both THP-treated and control rats were given an oral dose of [^{14}C]-CPZ (400 µg, 5 µCi) after a 12 h fast. [^{14}C]-CPZ hydrochloride (6.75 mCi/mmol) labelled in the ring was obtained from Applied Science Laboratories, Inc., State College, Pennsylvania. Two hours after the [^{14}C]-CPZ dose, the animals were anaesthetized with ether, an abdominal incision was made, and 5–10 ml of blood was drawn from the inferior vena cava into a tube containing heparin. The stomach was dissected out and its contents were drained into a tube; it was then cut open and washed three times with 0.01 N HCl, and the washings were collected. The stomach tissue was weighed and homogenized in 5 ml of 0.1 N HCl with a tissue homogenizer (Brinkman Polytron, Switzerland).

The brain was dissected out and washed three times with 0.01 N HCl and the washings were discarded. The brain was homogenized in 5 ml of 0.1 N HCl. The blood was centrifuged and the plasma was separated. Total radioactivity was assayed by adding aliquots of plasma (0.2 ml), stomach washings (0.5 ml), and stomach and brain homogenates (0.2 ml) solubilized in

Nuclear Chicago Solubilizer (NCS) to 10 ml of Triton X-toluene scintillation liquid and counting in a Packard Tri-Carb Scintillation Spectrometer. Counting efficiency for ^{14}C was 85–88%. All values of radioactivity were corrected for quenching and background.

Acute treatment

The distribution of radioactivity in the plasma, brain, and stomach 2 h after oral administration of [^{14}C]-CPZ in rats pretreated with one dose of THP (50 mg/kg) was studied following the procedure described above for chronic treatment. The THP was administered orally 1 h before an oral dose of [^{14}C]-CPZ. Matched controls were given water orally instead of THP before the dose of [^{14}C]-CPZ.

Measurement of gastric emptying

Male Sprague-Dawley rats were treated orally with THP (50 mg/kg) for one week, with matched controls receiving daily administration of water orally. After a 12 h fast, on the day of the experiment, the THP-treated and control rats were given [^{14}C]-labelled polyethylene glycol ([^{14}C]-PEG; 0.2 µCi) orally. Two hours later the rats were decapitated, and the stomach was dissected out, opened, and drained. The stomach cavity was washed with 0.01 N HCl three times, and the washings were collected for assay of radioactivity.

Effect of trihexyphenidyl on intestinal transport

The effect of THP on the transport of [^{14}C]-CPZ in everted sacs was studied under three different conditions as follows: (1) Effect of trihexyphenidyl when added directly to the incubation medium at the same time as [^{14}C]-CPZ was added. (2) Effect of 15 min preincubation of the everted sacs in THP solution, before testing the transport of [^{14}C]-CPZ. (3) Transport of [^{14}C]-CPZ by the everted sacs prepared from rats that were treated chronically with THP for 2 weeks.

General procedure of the everted sac experiment

Jejunal everted sacs were prepared from rats fasted for 12 h, by the method of Wilson & Wiseman (1954). The everted sac was filled with 0.5 ml of modified Krebs-bicarbonate buffer (pH 7.4) (Rivera-Calimlim, 1972) and securely closed with a ligature. The sacs, with appropriate treatment and substrate ([^{14}C]-CPZ) were incubated separately in 25 ml of Krebs-bicarbonate buffer (pH 7) for 30 min under 95% O_2 and 5% CO_2 at 100 oscillations per min in a Dubnoff metabolic shaking incubator. At the end of the incubation period the sacs were quickly removed and rinsed in 5–10 ml of ice-cold buffer. The serosal fluid was collected and measured, and the tissue was placed in a pre-weighed scintillation vial, dried in an oven at 105°C overnight and weighed. The tissues were solubilized in 1.5 ml NCS tissue solubilizer and the radioactivity was assayed in 10 ml of Triton X-100 toluene scintillation liquid in a Packard Liquid Scintillation Spectrometer. Counting efficiency was 88–90%. All counts were corrected for background and quenching.

The concentration of [^{14}C]-CPZ in the incubation medium was 1 mM (2 µCi per sac). The concentration of THP in both acute *in vitro* addition and preincubation was 1 mM. THP was not added to the incubation medium of the everted sacs prepared from THP chronically treated rats. The chronically treated rats were given oral THP (50 mg/kg) daily for 2 weeks; the last dose was given 1 h before they were killed.

Effect of trihexyphenidyl on the liver metabolism of chlorpromazine

Male Sprague-Dawley rats (120–150 g) were treated with oral THP daily, 50 mg/kg, for 1 week, with matched controls. At the end of the course of treatment the rats were fasted for 18 h and killed by decapitation, and the liver was dissected out. Approximately 1.5 g was homogenized in 4 volumes (4 times tissue weight) of 1.15% KCl–0.1M phosphate buffer (pH 7.4), the preparation being kept in ice throughout the procedure.

A sample of homogenate (0.5 ml) was added to 50 ml polystyrene tubes containing 1 ml of 0.1 M phosphate buffer (pH 7.4), 90 µmol of $MgCl_2$, 0.4 µmol of NADP, 15 nmol of glucose-6-phosphate, 1 i.u. glucose-6-phosphate dehydrogenase, and 0.5 µmol of [^{14}C]-CPZ (0.2 µCi). The total volume of incubation was 2 ml. The tubes were incubated at 37°C in a Dubnoff metabolic shaking incubator for 10 min at 100 oscillations/minute. After incubation, the chlorpromazine metabolites were separated from the parent compound by the solvent extraction technique of Curry, Derr & Maling (1970), with minor modifications. Samples (1 ml) were taken from the final heptane layers (after extraction of the heptane first with NaOH and then with sodium acetate buffer, pH 4.7) for assays of radioactivity, which represents unchanged chlorpromazine. Appropriate control samples were run simultaneously to determine the recovery of chlorpromazine, added to tissue homogenates, through the whole of the extraction procedure. This was found to be 57.7 ± 0.6%. All experimental values were, therefore, corrected for this recovery.

Results

Table 1 shows that rats treated chronically with THP for 2 weeks achieved significantly lower plasma levels

(20% of control) and lower brain levels (10% of control) of radioactivity from [^{14}C]-CPZ administered orally 2 h before killing, with significant retention of [^{14}C]-CPZ in the stomach contents of THP-treated rats. The radioactivity of the stomach contents was increased eight-fold in the THP-treated rats when compared to that of controls; radioactivity of the stomach wall in the treated rats was doubled. The same distribution pattern of oral [^{14}C]-CPZ was observed in rats that received only one dose of THP, although the changes observed were less marked than in the rats chronically treated with THP (Table 2). To test whether the stomach retention was due to the inhibition of gastric emptying or to gastric secretion of absorbed CPZ, gastric emptying was measured by the use of a marker, [^{14}C]-PEG, a substance which is neither adsorbed, metabolized or secreted. Results are shown in Table 3. The delay in gastric clearance of PEG indicates slow gastric emptying. Even 1 h after oral administration of [^{14}C]-PEG, 41–63% of the administered dose was recovered from the stomach of the rats pretreated with THP, but none was recovered from that of control rats.

Table 1 Distribution of orally administered [^{14}C]-chlorpromazine ([^{14}C]-CPZ) in rats chronically treated with trihexyphenidyl

Sample	*Controls* (n=8)	*Treated* (n=8)	P
Plasma (d min^{-1} ml^{-1})	2940.3±309	613.4±107	<0.001
Brain (%)*	0.068±0.008	0.007±0.005	<0.01
Stomach contents (%)*	3.08±0.76	26.85±5.87	<0.01
Stomach wall (%)*	3.24±0.75	7.63±0.31	<0.01

Values are expressed as mean±s.e. mean; *n*=number of rats. Significance of the difference was tested by the Student *t* test. Rats pretreated with oral trihexyphenidyl (50 mg/kg) and their controls were killed 2 h after an oral dose of [^{14}C]-CPZ (5 μCi). * Percent of the total dose given.

Table 2 Distribution of orally administered [^{14}C]-chlorpromazine ([^{14}C]-CPZ) in rats pretreated with one oral dose of trihexyphenidyl

Sample	*Controls* (n=8)	*Treated* (n=8)	P
Plasma (d min^{-1} ml^{-1})	3120±293	920±124	<0.01
Brain (%)*	0.078±0.01	0.034±0.007	<0.01
Stomach contents (%)*	3.52±0.34	14.85±3.87	<0.01
Stomach tissue (%)*	2.37±0.20	9.33±1.52	<0.01

Values are means±s.e mean; *n*=number of rats. Conditions are the same as for Table 1. * Percent of the total dose.

The possibility that THP also affects direct intestinal mucosal transport and liver metabolism of CPZ was studied. Table 4 shows that the mucosal transport of [^{14}C]-CPZ in the everted sacs exposed to THP in three different ways was not significantly different from that in matched controls. The 50 mg/kg dose of THP, which inhibited gastric emptying *in vivo*, did not inhibit mucosal transport of [^{14}C]-CPZ in everted sacs prepared from the same rat.

Table 5 shows that the metabolism of [^{14}C]-CPZ was not increased after incubation with liver homogenates prepared from rats treated with THP for 7 days.

Table 3 Gastric emptying as measured by [^{14}C]-polyethylene glycol ([^{14}C]-PEG)

Dose of THP (mg/kg)	*% [^{14}C]-PEG in stomach wash*	
	Control	*Treated*
50	0.1±0.06	41.3±1.3
100	0.1±0.06	63.3±17.3

Values are expressed as mean (±s.e. mean) percentage of recovery of total dose of [^{14}C]-PEG given orally, one hour after administration. Trihexyphenidyl (THP) was given orally one hour before [^{14}C]-PEG. The difference between control and treated on both doses was statistically significant (*P*<0.01). The difference between the treated groups given different doses was not significant.

Discussion

The lowering effect of trihexyphenidyl on plasma CPZ levels that has been observed in psychiatric patients

Table 4 Effect of trihexyphenidyl (THP) on transport of [^{14}C]-chlorpromazine ([^{14}C]-CPZ) in everted sacs

Experiment and incubation time	Recovery of CPZ in Serosal fluid (μmol/g) Control	Serosal fluid (μmol/g) Treated	Gut tissue (μmol/g) Control	Gut tissue (μmol/g) Treated
Addition of THP to medium				
15 min	0.63±0.18	0.49±0.07	27.6±2.6	28.6±4.0
30 min	0.62±0.13	0.61±0.21	42.2±2.64	38.0±1.83
Everted sacs prepared from chronically treated rats				
60 min	2.7±0.2	2.9±0.5	44.1±1.9	49.2±2.2
Everted sacs pre-incubated in THP for 15 min				
15 min	0.38±0.05	0.37±0.04	25.0±1.7	26.8±1.9
30 min	0.86±0.19	0.94±0.07	40.66±2.4	40.57±4.1

Values are expressed as mean±s.e. mean, $n=6$. Significance of the difference between TPH-treated and controls was tested by Student's t test and all P values were greater than 0.5

Table 5 Effect of trihexyphenidyl on [^{14}C]-chlorpromazine ([^{14}C]-CPZ) metabolism by rat liver *in vitro*

Source	μg CPZ metabolized per g liver tissue
Treated ($n=8$)	574.8±43.0
Control ($n=8$)	512.1±32.7

Values are the mean±s.e. mean; n=number of rats. Significance of the difference was tested by the Student t test, $P>0.5$. Samples were run in duplicate.

given both drugs was shown to be reproducible in rats in the present study. At the time when low plasma levels of CPZ were observed in THP-treated rats, the brain levels were also reduced, whereas in the stomach the CPZ levels were significantly higher than in control rats. This pattern of tissue distribution of CPZ (Table 1) in rats treated with THP was observed in both the chronic and the acute experiments and suggested that the lower plasma levels of CPZ in treated rats may be due to inhibition of gastric emptying resulting from the antiacetylcholine property of THP. Rivera-Calimlim, Dujovne, Morgan, Lasagna & Bianchine (1971) have observed that absorption of certain drugs is affected by inhibition of gastric emptying, caused either by increase in gastric acidity or by antiacetylcholine agents such as imipramine (Morgan, Nathan & Rivera-Calimlim, 1972; Morgan, Nathan, Rivera-Calimlim & Trabert, 1975) and Adjepon-Yamah, Scott & Prescott (1973) have reported a striking delay in the absorption of oral lignocaine in patients treated with atropine prior to laparoscopy. Similarly, Consolo, Morselli, Zaccala & Garratini (1970) have shown that desmethyl imipramine delays the absorption of phenylbutazone. Nimmo, Heading, Tothill & Prescott (1973) demonstrated the inhibitory effect of propantheline, an antiacetylcholine agent, on absorption of paracetamol.

The effect of THP on gastric emptying was, therefore, studied by the use of radioactive PEG as a marker and the results showed conclusively that THP significantly delayed the gastric emptying. The other possible mechanisms whereby THP can lower plasma levels of CPZ are a direct inhibition of THP of intestinal absorption or a stimulation by THP of the liver metabolism of CPZ. THP has no effect on intestinal tissue uptake and mucosal transport of CPZ in everted sacs. Likewise, the *in vitro* metabolism of CPZ by liver homogenates from rats treated chronically with THP did not change significantly when compared with the controls. It can, therefore, be concluded from this study that the lower plasma levels of CPZ observed in rats when CPZ is administered with THP is due to inhibition of gastric emptying, and this may also be the mechanism involved in man.

This work was supported by grant No. MH 24188 from the U.S. Public Health Service. I am grateful to Professor L. Lasagna for his interest and advice in this work, and to Susan Kelvie and A. Siracusa for their skilled technical assistance.

References

ADJEPON-YAMAH, K.K., SCOTT, D.B. & PRESCOTT, L.F. (1973). Impaired absorption and metabolism of oral lignocaine in patients undergoing laparoscopy. *Br. J. Anesth.*, **45**, 143–147.

CONSOLO, S., MORSELLI, P.L., ZACCALA, M. & GARRATINI, S. (1970). Delayed absorption of phenylbutazone caused by desmethyl imipramine in humans. *Eur. J. Pharmac.*, **10**, 239–242.

CURRY, S.H., DERR, J.E & MALING, H.M. (1970). The physiological disposition of chlorpromazine in the rat and dog. *Proc. Soc exp. Biol. Med.*, **134**, 314–318.

MORGAN, J.P., NATHAN, G & RIVERA-CALIMLIM, L. (1972). Imipramine-caused interference with levodopa absorption in the rat. (Abstract) *Fifth International Congress on Pharmacology*, No. 963, p. 167.

MORGAN, J.P., NATHAN, G., RIVERA-CALIMLIM, L & TRABERT, N (1975). Imipramine-caused interference with levodopa absorption from the gastrointestinal tract in rats *J. Pharmac. exp. Ther.*, **192**, 451–457.

NIMMO, J., HEADING, R.C., TOTHILL, P & PRESCOTT, L.F. (1973). Pharmacological modification of gastric emptying· Effects of propantheline and metaclopropamide on paracetamol absorption. *Br. Med. J.*, **I**, 587–789.

RIVERA-CALIMLIM, L (1972). Effect of chronic drug treatment on intestinal membrane transport of ^{14}C-L-DOPA. *Br. J. Pharmac.*, **46**, 708–713.

RIVERA-CALIMLIM, L, CASTAÑEDA, L & LASAGNA, L. (1973). Effects of mode of management on plasma chlorpromazine in psychiatric patients. *Clin. Pharmac. Ther.*, **14**, 978–986.

RIVERA-CALIMLIM, L, DUJOVNE, C.A., MORGAN, J.P., LASAGNA, L. & BIANCHINE, J.R (1971). Absorption and metabolism of L-dopa by the human stomach. *Eur. J. clin. Invest.*, **1**, 313–320.

RIVERA-CALIMLIM, L, NASRALLAH, H., STRAUSS, J. & LASAGNA, L. (1975) Clinical response and plasma levels; effect of dose, dosage schedules and drug interaction on plasma chlorpromazine level. *Am. J Psychiat.*, (in press).

SINGH, M.M. & KAY, S.R (1974). Therapeutic antagonism between neuroleptics and anticholinergic antiparkinsonism agents in schizophrenia. *J. Bronx State Hospital*, **2**, 8–20.

SINGH, M M. & KAY, S R. (1975). Therapeutic reversal with benztropine in schizophrenics–practical and theoretical significance. *J. nerv. ment. Dis.*, **60**, 258–266.

SINGH, M.M & SMITH, J. (1973). Reversal of some therapeutic effects of an antipsychotic agent by an antiparkinson drug. *J. nerv. ment. Dis.*, **157**, 50–57.

WILSON, T.H. & WISEMAN, G (1954). Metabolic activity of the small intestine of the rat and golden hamster. *J. Physiol. Lond.*, **123**, 126–130.

(Received August 29, 1975.
Revised November 19, 1975.)

Br. J. Pharmac. (1976), 56, 307–311

BENZODIAZEPINES AND CENTRAL GLYCINE RECEPTORS

D.R. CURTIS, C.J.A. GAME & D. LODGE
Department of Pharmacology, Australian National University, Canberra, Australia

1 In cats, anaesthetized with pentobarbitone, intravenous diazepam (minimum dose 3.0 mg/kg) enhanced dorsal root potentials but did not significantly diminish the reduction by electrophoretic strychnine of the inhibitory action of electrophoretic glycine on dorsal horn interneurones.

2 In mice, intraperitoneal diazepam (2.5 mg/kg) had no appreciable effect on the potency of strychnine as a convulsant, although providing some protection against bicuculline.

3 These observations, together with the failure of chlordiazepoxide to either inhibit the firing of spinal interneurones or reduce antagonism between strychnine and glycine when administered locally, provide no support for the interaction between benzodiazepines and mammalian central glycine receptors which has been proposed on the basis of *in vitro* studies of strychnine binding.

Introduction

The inhibitory effect of glycine upon spinal and supraspinal neurones in the mammalian central nervous system is antagonized by strychnine (Curtis & Johnston, 1974). A number of recent publications, reviewed by Snyder (1975), have been concerned with the detection of postsynaptic glycine receptors on the basis of *in vitro* binding of tritiated strychnine to synaptic membrane fractions of central nervous tissue of rats (and monkeys, Young & Snyder, 1973). 'Specific' binding of the alkaloid to glycine receptors was defined as the amount of bound strychnine displaced by 10 mM glycine, and a remarkable correlation was found between the regional distribution of binding sites, the levels of endogenous glycine and the high affinity glycine uptake systems. Furthermore, strychnine binding was reduced by amino acids such as β-alanine, α-alanine, taurine and proline, considered 'glycine-like' on the basis of the sensitivity of their central inhibitory effects to strychnine (Curtis & Johnston, 1974), but not by amino acids structurally related to γ-aminobutyric acid (GABA).

A correlation between the 'clinical efficacy' of a series of benzodiazepines and the ability of these compounds to reduce the binding of strychnine to glycine receptors has led to the proposal that benzodiazepines, widely used therapeutically to control anxiety, seizures and muscle spasticity, 'exert their pharmacological activities by interacting with the glycine receptor' (Young, Zukin & Snyder, 1974; Snyder, 1975). Since the depressant effects of diazepam on spinal reflexes have largely been interpreted in terms of disturbed 'presynaptic' inhibition, a process involving GABA as a transmitter (see Schmidt, Vogel & Zimmermann, 1967; Schmidt, 1971; Polc, Möhler & Haefely, 1974; Costa, Guidotti, Mao & Suria, 1975), a study has been made in the cat and the mouse of the possible interaction between diazepam, strychnine and glycine *in vivo*. As a consequence no direct support can be offered to the proposal that benzodiazepines interact with glycine receptors in the spinal cord.

Methods

Cat spinal cord

Standard methods were used to record extracellular action potentials from dorsal horn interneurones of lumbar segments of cats (body temperature 37–38°C) anaesthetized with pentobarbitone sodium, using the centre barrel of seven barrel micropipettes as the recording electrode (Curtis, Duggan, Felix & Johnston, 1971). The outer barrels of these micropipettes contained aqueous solutions of DL-homocysteate (0.2 M, NaOH, pH 7.5); glycine (0.5 M, HCl, pH 3); GABA (0.5 M, HCl, pH 3); strychnine hydrochloride (0.002 M in 0.165 M NaCl); bicuculline methochloride (0.01 M in 0.165 M NaCl) and chlordiazepoxide hydrochloride (0.2 M), from which pharmacologically active ions were ejected electrophoretically.

Records were obtained from one dorsal horn interneurone in each of 3 cats before and for up to 30 min after the intravenous administration of diazepam. The commercially available solution of diazepam (Valium, Roche; 10 mg/2 ml benzoate buffer) was diluted with 2 ml ethanol and 16 ml of 0.165M NaCl to provide a solution containing diazepam 0.5 mg/ml. Continuous monitoring of the amplitude of extracellular action potentials, and of the sensitivity of the neurones to DL-homocysteate, GABA and bicuculline methochloride, as well as to glycine and strychnine, provided a control for possible alterations in the distance between the neurones and micropipettes as a cause of changes in glycine or strychnine effects.

Dorsal root potentials were recorded from a caudal portion of the sixth lumbar dorsal root, in response to electrical stimulation of either hindlimb peripheral nerves (tibial, common peroneal) or the seventh lumbar dorsal root. In the doses used, diazepam reduced the systemic blood pressure by less than 10–15 mmHg; in all 3 animals dorsal root potentials were increased in amplitude and duration, and in view of the results of Polc *et al.* (1974) control experiments using the solvent alone were not performed.

Mice

In order to determine whether diazepam would protect against strychnine and bicuculline-induced convulsions and death, dose-response curves were prepared for these two drugs with and without the prior administration of diazepam (2.5 mg/kg). Four or five groups of 9 mice (body weight 30–37.5 g) were used to plot each curve. The solutions of strychnine (0.5 mg/ml), bicuculline (1 mg/ml) and diazepam (5 mg/ml, Valium; Roche) were administered intraperitoneally. Diazepam was injected 30 min before the administration of the convulsant, and mice were observed for a further 30 minutes. The convulsant end point used was either bilateral extensor spasms of the hind legs or opisthotonos. Dose-response curves were prepared by plotting linearly the number of animals convulsing or dying in each group against the dose of convulsant administered.

Results

Spinal interneurones

In preliminary experiments chlordiazepoxide could rarely be successfully administered electrophoretically, micropipettes containing solutions of this substance becoming 'noisy' with increased electrical resistance during the passage of even small electrophoretic currents. Chlordiazepoxide, ejected with currents of 2–5 nA did not reduce the firing of 5 interneurones, although glycine ejected with similar or even smaller currents clearly inhibited firing.

Attempts were made to administer chlordiazepoxide hydrochloride (0.1 M; 0.2 M; and 0.05 M in 0.165 M NaCl) by pressure (to 300 mmHg), either from one barrel of a 7 barrel micropipette or from another pipette (orifice 10–20 µm) cemented alongside. Although 3 of these neurones were readily excited by DL-homocysteate ejected with similar or even lower pressures from an adjacent barrel, the unreliability of this method of administration prevented a definite conclusion from the observed failure of chlordiazepoxide (3 neurones 0.05 M, 5 neurones 0.1 M, 1 neurone 0.2 M) either to reduce the effectiveness of strychnine as a glycine antagonist or to influence the firing of 9 neurones.

Results from one of the three experiments in which diazepam was administered intravenously are illustrated in Figure 1. In these experiments glycine and GABA were administered in amounts which did not completely suppress neuronal firing. Furthermore, the currents selected to administer strychnine and bicuculline methochloride were such that complete and rapid abolition of the actions of glycine and GABA did not occur. Thus both the rate of onset and the degree of antagonism could be used as parameters to assess the influence of diazepam. No consistent reductions in firing rate occurred after these doses of diazepam.

Control observations (Figure 1a) were made during excitation of the neurone by DL-homocysteate (8.5 nA). Strychnine (7 nA) progressively reduced the action of glycine (0 nA, removal of retaining current), that of the third dose being 27% of the effect observed beforehand. Similarly, and not illustrated, bicuculline methochloride (20 nA for 4.5 min) reduced the effectiveness of GABA to 30%. Seven minutes after intravenous diazepam (0.5 mg/kg) the effects of glycine, GABA, strychnine and bicuculline were unchanged. Twenty minutes later a further dose of 1.0 mg/kg was administered, and 8 min later the tracings of Figure 1b were recorded. The currents used to eject amino acids had been altered slightly to obtain responses similar to those of Figure 1a. Nevertheless the rate of antagonism of the inhibitory action of glycine by strychnine appeared not to be altered, and the effect of the third dose of glycine was approximately 17% of that occurring before strychnine was administered. At this time the dorsal root potential recorded from the caudal portion of the sixth lumbar dorsal root in response to stimulation of the seventh dorsal root was increased in amplitude and duration. The total area of the dorsal root potential was increased by 53% (see also Schmidt *et al.*, 1967; Polc *et al.*, 1974), and the short latency dorsal root reflex component was also enhanced.

Subsequently a further 1.5 mg/kg of diazepam was administered and 11 min later strychnine (7 nA) again reduced the action of glycine to 23% of that seen prior to strychnine. There was thus no evidence provided in this preparation that diazepam (3 mg/kg) reduced the effectiveness of strychnine as a glycine antagonist, and similar results were obtained in the other two cats with

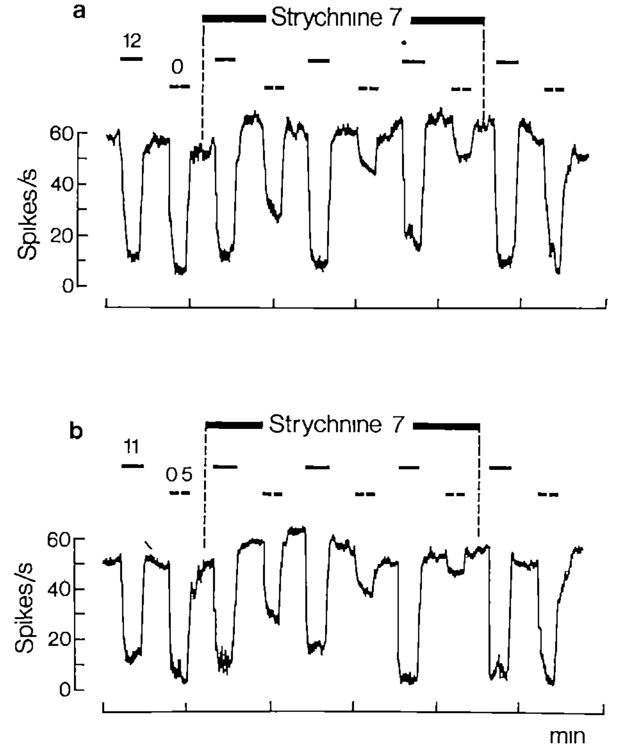


Figure 1 The effect of strychnine (a) before and (b) after the intravenous administration of diazepam, 1 5 mg/kg The tracings plot the firing rate of a spinal dorsal horn interneurone excited by DL-homocysteate (a) 8 5 nA, (b) 9 nA. The firing was inhibited by periodic ejections of γ-aminobutyric acid (GABA, 7 s) and glycine (8 s) as indicated by the horizontal solid (GABA) and broken lines (glycine) and electrophoretic currents. Strychnine was administered with a current of 7 nA from a solution of strychnine hydrochloride 0.002 M in 0 165M NaCl

increasing doses of diazepam to 3.0 and 3.85 mg/kg respectively.

In one of these cats the effect of bicuculline methochloride as a GABA antagonist appeared to be reduced by 1.5 mg/kg diazepam. However, the failure of increasing doses of diazepam (to 3 mg/kg) to decrease progressively the action of bicuculline methochloride, and the finding in the other two cats that diazepam did not significantly reduce the effect of bicuculline methochloride on the inhibitory action of GABA, suggests that the observation made in one animal probably arose from the vagaries of the techniques used rather than from an interaction between diazepam and bicuculline receptors.

Mice

Dose-response curves showing the convulsant activity of intraperitoneal strychnine and bicuculline are plotted in Figure 2. Diazepam had no appreciable effect on the potency of strychnine (Figure 2a) as a convulsant whereas it did afford protection against bicuculline-induced convulsions (Figure 2b). The most significant group was that receiving 6.3 mg/kg of bicuculline which convulsed 8 of 9 control mice and only 2 of 9 mice injected previously with 2.5 mg/kg diazepam. Using this data plotted on log-dose/probit scales the CD_{50} for bicuculline was 4.2 mg/kg in the control group and 8.5 mg/kg following diazepam; the CD_{50} for strychnine was 1.54 mg/kg in the control and 1.46 mg/kg in the diazepam-injected group.

The convulsant end points were easy to recognize with strychnine, but were slightly more difficult with bicuculline, especially since the convulsions appeared to be somewhat less severe in the diazepam-treated bicuculline animals. It was quite clear, however, when plotting dose-response curves for death (Figure 2c and d), rather than for convulsions, that diazepam offered far more protection against bicuculline than against strychnine.

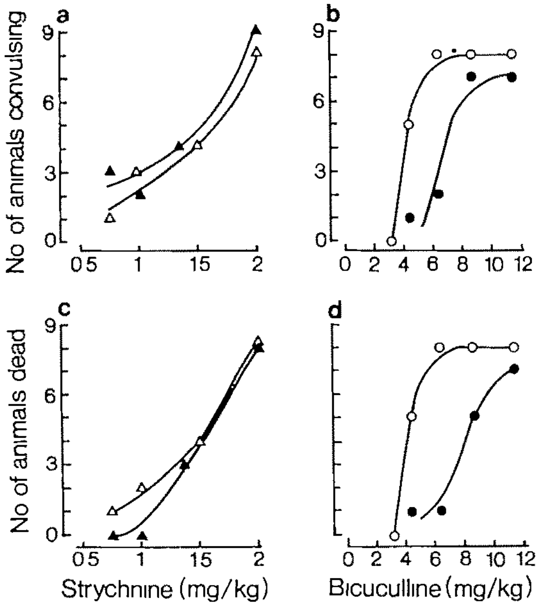


Figure 2 Effect of diazepam on the potency of strychnine and bicuculline as convulsants (a, b) and lethal agents (c, d) in mice. The curves were plotted from data obtained from groups of 9 mice following the intraperitoneal injection of (a, c) strychnine and (b, d) bicuculline with (▲, ●) and without (△, ○) the administration of diazepam (2.5 mg/kg) 30 min previously. Ordinates: number of animals convulsing (a, b) or dying (c, d) in each group Abscissae dose of convulsant, mg/kg.

Discussion

Despite uncertainties regarding the adequacy of electrophoretic or pressure ejection, no glycine-like effects of chlordiazepoxide, or impairment of the antagonism of glycine by strychnine, were observed in a small number of experiments. Furthermore, systemically administered diazepam, in relatively large doses which were sufficient to enhance and prolong dorsal root potentials, had no significant effect on the sensitivity of spinal interneurones to either glycine or GABA, and did not influence the effectiveness of strychnine or bicuculline methochloride as selective antagonists of these two amino acids. The total dose of diazepam tested was approximately 30 times that used clinically, for which the claim has been made that central nervous system and blood concentrations are similar to those required to displace strychnine from binding sites (Young *et al.*, 1974).

Although relatively high doses of diazepam (16 mg/kg orally) have been reported to inhibit strychnine convulsions in mice (see Zbinden & Randall, 1967), Costa *et al.* (1975) have recently reported that diazepam and chlordiazepoxide antagonize seizures induced in mice by picrotoxin more effectively than those produced by strychnine. The present results with mice indicate that at a dose level of 2.5 mg/kg diazepam had little or no influence on either the convulsant or lethal effects of strychnine, although providing some protection against these effects of bicuculline. Similar results have been reported by Schlosser, Zavatsky, Kappel & Sigg (1973). The failure of systemic diazepam to modify at a single cell level the effects of GABA or bicuculline suggests that the influence of benzodiazepines on GABA-mediated inhibitory processes may be more complex than mere interaction at GABA postsynaptic receptors.

The affinity of strychnine for its binding site *in vitro* is three orders of magnitude greater than that of glycine (Young & Snyder, 1973), and under the experimental conditions used (rat crude synaptic membrane fractions, 2 nM strychnine, 4°C) glycine and diazepam were of comparable potency in reducing strychnine binding, half maximum inhibition occurring with concentrations of 25 μM and 26 μM respectively (Young *et al.*, 1974). Thus if diazepam mimics the postsynaptic action of glycine (or strychnine) and interferes with strychnine binding the present investigation might have been expected to have revealed a modification in the effectiveness of strychnine as a glycine antagonist and as a convulsant. That this apparently is not so suggests that the receptors detected by *in vitro* strychnine binding may not be those directly involved in the inhibitory action of glycine. This conclusion may have implications for the interpretation of data regarding other central neurotransmitter receptors which depends upon membrane binding, under undoubtedly non-physiological conditions, rather than on the initiation or antagonism of a physiological response.

The authors wish to thank Dr R.J. Mulhearn, Roche Products Pty. Ltd. for samples of benzodiazepines, and Mrs P. Searle for technical assistance.

References

COSTA, E., GUIDOTTI, A., MAO, C.C. & SURIA, A. (1975). New concepts on the mechanism of action of benzodiazepines. *Life Sci. Oxford.*, **17**, 167–186.

CURTIS, D.R., DUGGAN, A.W., FELIX, D. & JOHNSTON, G.A.R. (1971). Bicuculline, an antagonist of GABA and synaptic inhibition in the spinal cord. *Brain Res.*, **32**, 69–96.

CURTIS, D.R. & JOHNSTON, G.A.R. (1974). Amino acid transmitters in the mammalian central nervous system. *Ergebn. Physiol.*, **69**, 97–188.

POLC, R., MÖHLER, H. & HAEFELY, W. (1974). The effect of diazepam on spinal cord activities: possible sites and mechanisms of action. *Naunyn-Schmiedeberg's Arch. Pharmac.*, **284**, 319–337.

SCHLOSSER, W., ZAVATSKY, E., KAPPEL, B. & SIGG, E.B. (1973) Antagonism of bicuculline and Ro 5-3663 by diazepam. *Pharmacologist*, **15**, 162.

SCHMIDT, R.F (1971). Presynaptic inhibition in the vertebrate central nervous system. *Ergebn. Physiol.*, **63**, 20–101.

SCHMIDT, R.F., VOGEL, M E & ZIMMERMANN, M. (1967). Die Wirkung von Diazepam auf die prasynaptische Hemmung und andere Ruckenmarksreflexe. *Naunyn-Schmiedeberg's Arch. Pharmac. Path.*, **258**, 69–82.

SNYDER, S.H (1975). The glycine synaptic receptor in the mammalian central nervous system. *Br. J. Pharmac.*, **53**, 473–484.

YOUNG, A.B & SNYDER, S.H. (1973). Strychnine binding associated with central nervous glycine receptors. *Proc. natn. Acad. Sci. U.S.A.*, **70**, 2832–2836.

YOUNG, A B, ZUKIN, S R. & SNYDER, S.H. (1974). Interaction of benzodiazepines with central nervous glycine receptors. possible mechanism of action. *Proc. natn. Acad. Sci. U.S.A.*, **71**, 2246–2250.

ZBINDEN, G & RANDALL, L.O (1967). Pharmacology of benzodiazepines: laboratory and clinical correlations. *Adv. Pharmac*, **5**, 213–291.

(Received September 1, 1975.
Revised November 11, 1975.)

Br. J. Pharmac. (1976), **56**, 313–316

STUDIES ON THE RELATIVE EFFECTS OF PROSTAGLANDINS, BRADYKININ, 5-HYDROXYTRYPTAMINE AND HISTAMINE ON THE SYNOVIAL MICROCIRCULATION IN DOGS

W.C. DICK & D.M. GRENNAN
The Centre for Rheumatic Diseases and University Department of Medicine, Royal Infirmary, Glasgow, and the Wellcome Surgical Research Institute, Glasgow
I.J. ZEITLIN
Department of Physiology and Pharmacology, University of Strathclyde, George Street, Glasgow G1 1XW

1 The relative effects of prostaglandins E_1, E_2, $F_{1\alpha}$, $F_{2\alpha}$, bradykinin, histamine and 5-hydroxytryptamine (5-HT) on the canine synovial microcirculation were investigated using the rate of clearance of radioactive xenon (^{133}Xe) as an index of synovial perfusion.

2 All the compounds tested, except prostaglandin $F_{1\alpha}$, produced a vasodilator effect. The descending order of potency of the active compounds was (i) prostaglandin E_1, (ii) prostaglandin E_2 and bradykinin, (iii) histamine and 5-HT, (iv) prostaglandin $F_{2\alpha}$. The most potent compound tested, prostaglandin E_1 produced an effect in nanogram amounts in each joint.

3 Prostaglandin $F_{2\alpha}$ has been reported to have an anti-inflammatory action; however no evidence was found of antagonism of prostaglandin E_1 by two dose levels of prostaglandin $F_{2\alpha}$.

4 Preliminary studies showed that threshold doses of prostaglandin E_1 did not potentiate the vasodilator action of threshold or sub-threshold doses of bradykinin in the dog synovium.

Introduction

Current concepts of the pathophysiology of clinical joint inflammation include a role for the various chemical mediators of inflammation (Austen, 1972). Recently most interest has been displayed in the prostaglandins. Prostaglandin E-like activity has been found in increased amounts in the synovial effusions of experimentally induced arthritis in rabbits (Blackham, Farmer, Radziwonik & Westwich, 1974). Prostaglandins E and F have been shown to be present in rheumatoid synovial effusions (Giroud, Velo, Dunn, Timsit & Willoughby, 1974; Higgs, Vane, Hart & Wojtulewski, 1974). Kinins have also been detected in increased amounts in the synovial fluid of various arthritides (Melmon, Webster, Goldfinger & Seegmiller, 1967). Histamine is present in both normal and abnormal human synovium (Roth, Polley & Cohn, 1964; Zachariae & Zachariae, 1965) but this amine and 5-hydroxytryptamine (5-HT) have attracted less attention from investigators. The effects of these substances have been investigated on a variety of vascular beds (Majno & Pallade, 1961; Lewis, 1964; Zweifach, 1964; Viguera & Sunahara, 1969). As a help to the understanding of the relative importance of the various mediators in the pathogenesis of joint inflammation, we have investigated their activities on the normal synovial microcirculation. In these experiments we have compared the effects of prostaglandins E_1, E_2, $F_{1\alpha}$, and $F_{2\alpha}$, bradykinin, 5-HT and histamine on canine synovial perfusion, using the half-life ($T\frac{1}{2}$) of ^{133}Xenon clearance following intra-articular injection of the isotope.

Methods

These studies were carried out on adult mongrel dogs weighing between 20 and 30 kg and anaesthetized with thiopentone, nitrous oxide, O_2 and less than 1% halothane. Blood pressure and blood gases were monitored and maintained constant throughout all experiments.

Synovial blood flow was monitored indirectly by calculating the half-life ($T\frac{1}{2}$ min) of the clearance of the inert gas, radioactive xenon (^{133}Xe), from the knee and elbow joints following intra-articular injection of 0.1 ml of the gas dissolved in sterile 0.9% w/v NaCl solution (saline). This method provides an index of synovial perfusion (Dick, St. Onge, Gillespie, Downie, Nuki, Gordon, Whaley, Boyle & Buchanan, 1970), a decrease in the $T\frac{1}{2}$ value denoting a vasodilator response and an increase denoting a vasoconstrictor

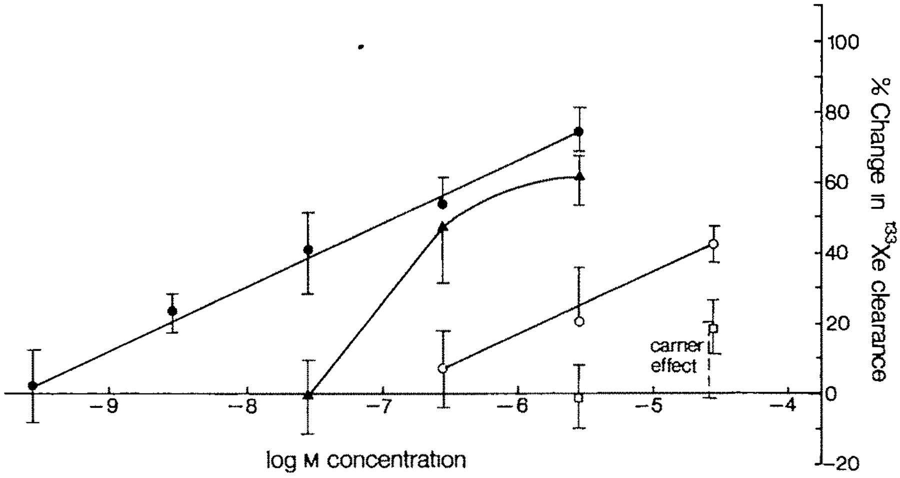


Figure 1 The effects of prostaglandin E_1 (●), E_2 (▲), $F_{1\alpha}$(□) and $F_{2\alpha}$ (○) on percentage change of $T\frac{1}{2}$ values of ^{133}Xe clearance ($n \geq 5$). Vertical lines show s.e. mean

response. There is considerable individual variation in the base-line xenon clearance from individual joints but repeated studies on the same joint have demonstrated acceptable reproducibility, provided strict attention is paid to critical aspects of the method. (Dick *et al.*, 1970). Clearance of the isotope was followed using a thallium activated sodium iodide scintillation crystal connected to a pulse height analyser, photomultiplier and rate meter recording on a potentiometric chart recorder. In each experiment a base-line recording from 15 to 20 min was taken, followed by further 15 to 20 min recordings following intra-articular injection of the agents used in the various doses (prostaglandins E_1 and E_2 0.1–100 ng; prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ 1 ng–1 µg; histamine and 5-HT 10 ng–1 µg). The molecular weights of the various compounds tested are prostaglandin E_1 354, E_2 352, $F_{1\alpha}$ 356, $F_{2\alpha}$ 354, histamine acid phosphate 307, 5-hydroxytryptamine creatinine sulphate 405, and bradykinin hydrochloride 1060. All dose levels of each compound were tested five or more times. Statistical significance of alterations in response was determined using the Mann-Whitney *U* test.

The following drugs were used: prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ (Upjohn Co., Kalamazoo, U.S.A.); bradykinin hydrochloride and 5-hydroxytryptamine creatinine sulphate (Sandoz Products Ltd.) and histamine acid phosphate (Evans Medical). Prostaglandins were dissolved in ethanol (1 mg/ml) and diluted further in saline.

Results

Figure 1 compares the dose-response curves for prostaglandins E_1, E_2, $F_{1\alpha}$, and $F_{2\alpha}$. None of the prostaglandin $F_{1\alpha}$ effects shown differed significantly from the effects of the diluent when analyzed by the Mann-Whitney U test.

Prostaglandin $F_{2\alpha}$ produced a significant vasodilator effect in high dosage only and statistical analysis showed no difference between the carrier effect and all but the highest dose shown in Figure 1. Prostaglandins E_1 and E_2 were both extremely potent and produced dose-related vasodilator effects with prostaglandin E_1 being the more potent.

Figure 2 shows that the effects of bradykinin, 5-HT and histamine fell between those of prostaglandins E_1 and $F_{2\alpha}$, the order of potency on a molar basis being prostaglandin E_1, bradykinin, histamine and 5-HT, and then prostaglandin $F_{2\alpha}$.

The lowest effective molar concentrations of the various compounds are shown in Table 1.

Table 1 Approximate threshold concentrations of vasodilators producing change in $T\frac{1}{2}$ values of ^{133}Xe clearances

Mediator	*Threshold concentration (µM)*
Prostaglandin E_1	0.028
Prostaglandin E_2	0.28
Bradykinin	0.1
5-Hydroxytryptamine	2.4
Histamine	3.2
Prostaglandin $F_{2\alpha}$	28.0
Prostaglandin $F_{1\alpha}$	No effect at concentrations up to 28.0

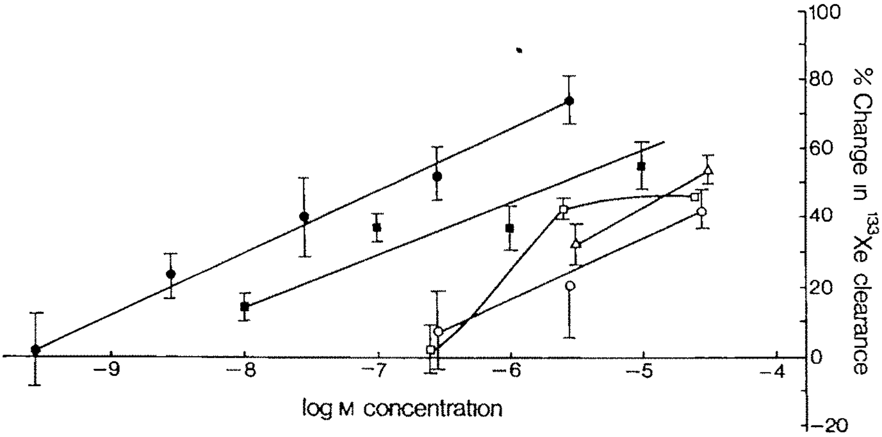


Figure 2 The effects of prostaglandin E_1 (●), $F_{2\alpha}$ (○), bradykinin (■), histamine (△) and 5-hydroxytryptamine (□) on $T\frac{1}{2}$ value of ^{133}Xe clearance ($n \geqslant 5$) Vertical lines show s.e mean.

Prostaglandin E_1 was the most potent, prostaglandin E_2 and bradykinin approximately 5–10 times less effective than prostaglandin E_1, 5-HT and histamine approximately 50–100 times less effective than prostaglandin E_1 and prostaglandin $F_{2\alpha}$ approximately 500–1000 times less effective than E_1.

The mean percentage changes in ^{133}Xe clearance produced by prostaglandin E_1 28 nM given after pretreatment of the joint with 28 nM or 2.8 μM prostaglandin $F_{2\alpha}$ were 35.5% and 47.1% respectively. The responses were similar to the mean of 39.7% brought about by this dose of prostaglandin E_1 alone.

The mean percentage change in ^{133}Xe clearance produced when bradykinin 0.1 μM was injected just after pretreatment of the joint with prostaglandin E_1 28 nM was 37.2% ± 9.9 which is not detectably different from the change produced by prostaglandin E_1 when injected alone (39.7%). The injection of the subthreshold dose of 10 nM bradykinin after the subthreshold dose of 2.8 nM prostaglandin E_1 produced no significant average vasodilator response (−10% ± 7.3).

Discussion

In these experiments the effects of various known inflammatory mediators on the normal synovial blood flow have been examined. Prostaglandin E_1 was the most potent vasodilator substance tested producing a response in nanogram amounts. Prostaglandin E_2 was also a potent vasodilator, approximately 5–10 times less potent than prostaglandin E_1. These results are of interest in that prostaglandin E_1 is reported to be the main prostaglandin in the synovial fluid of inflamed joints both in experimental animals (Blackham *et al.*, 1974) and in rheumatoid arthritis (Higgs *et al.*, 1974).

Bradykinin was about as active on a molar basis as prostaglandin E_2 in producing an effect on synovial perfusion. Histamine and 5-HT also produced a definite increase in synovial perfusion in certain doses. Their potency lay between that of prostaglandin E_2 and bradykinin on the one hand and prostaglandin $F_{2\alpha}$ on the other.

Prostaglandin $F_{2\alpha}$ produced a significant vasodilator effect in microgram dosage only and is thus a much weaker vasodilator than bradykinin, prostaglandin E_1 and E_2. Prostaglandin $F_{2\alpha}$ has been shown to inhibit the vascular permeability increasing action of prostaglandin E_1 (Willoughby, 1968) and Giroud *et al.* (1974) have suggested that prostaglandin $F_{2\alpha}$ may be associated with the waning of the naturally occurring inflammatory process and may in fact act as an anti-inflammatory mediator. We looked for an antagonistic effect of two doses of prostaglandin $F_{2\alpha}$ on the vasodilator effect of prostaglandin E_1 in the synovium but found no evidence for this.

Prostaglandin E_1 can in certain situations, potentiate bradykinin both in its pain producing effects (Ferreira, 1972) and in its effects on increasing vascular permeability (Williams & Morley, 1973). The synovial vasculature appears sensitive to small amounts of both prostaglandin E_1 and bradykinin but so far we have been unable to demonstrate potentiation by prostaglandin E_1 of the effects on synovial blood flow of a threshold or subthreshold dose of bradykinin.

We are grateful for technical assistance to Mr Littlejohn and staff of the Wellcome Surgical Research Institute, Glasgow, and for financial assistance from the National Fund for Research into Crippling Diseases. We would like to thank the following for generous gifts of drugs: Dr J E. Pike, Upjohn Co., Kalamazoo, U.S.A. for prostaglandins and Miss E.R.

Nimmo, Sandoz Products Ltd. for bradykinin and 5-hydroxytryptamine This work was carried out by D.M.G. in partial fulfilment of requirements for the degree of Ph.D. at the University of Strathclyde.

References

AUSTEN, K.F (1972). The acute inflammatory response in arthritis and allied conditions. In *Arthritis and Allied Conditions,* ed. Hollander, J.L., p. 136. Philadelphia: Lea and Febiger.

BLACKHAM, A, FARMER, J.B., RADZIWONIK, H. & WESTWICK, J. (1974) The role of prostaglandins in rabbit mono-articular arthritis. *Br. J. Pharmac.,* **51**, 35–44

DICK, W C, ST. ONGE, R A., GILLESPIE, F.C, DOWNIE, W W, NUKI, G, WHALEY, K., BOYLE, J A. & BUCHANAN, W.W. (1970). Derivation of knee joint synovial perfusion using the ^{133}Xe clearance technique. *Ann. Rheum. Dis.,* **29**, 131–134.

FERREIRA, S H. (1972). Prostaglandins, aspirin-like drugs and analgesics. *Nature, New Biol.,* **240**, 200–203.

GIROUD, J P, VELO, G.P., DUNN, C.J., TIMSIT, J & WILLOUGHBY, D.A (1974). Distribution of prostaglandins in inflammatory exudate. In *Future Trends in Inflammation,* p. 19. Padua & London, Piccin Med. Books.

HIGGS, G A., VANE, J R., HART, F D & WOJTULEWSKI, J A (1974). Effects of anti-inflammatory drugs on prostaglandins in rheumatoid arthritis. In *Prostaglandin Synthetase Inhibitors,* ed. Robinson, H.J. & Vane, J.R., pp. 165–173. New York: Raven Press.

LEWIS, G.P. (1964). Plasma kinins and other vasoactive compounds in acute inflammation. *Ann. N.Y. Acad. Sci.,* **116**, 847–854.

MAJNO, G & PALLADE, G.E. (1961). Studies on inflammation. 1. Effect of histamine, serotonin on vascular permeability. An electron-microscope study. *J. biophys. biochem. Cytol.,* **11**, 571–605.

MELMON, K L., WEBSTER, M.E., GOLDFINGER, S E. & SEEGMILLER, J.E. (1967). The presence of a kinin in inflammatory synovial effusion from arthritides of various aetiologies. *Arth. Rheum.,* **10**, 13–19.

ROTH, S.H, POLLEY, H F & COHN, C.F (1974). The activity of histamine and the related enzyme systems in human synovial membrane of fluid. *Arthritis and Rheum.,* **7**, 749–750.

VIGUERA, M F. & SUNAHARA, F.A. (1969). Microcirculatory effects of prostaglandins. *Canad. J. Physiol. Pharmac.,* **41**, 627–634.

WILLIAMS, T J & MORLEY, J (1974). Measurement of extravasation of plasma protein in inflammatory response in guinea-pig skin using a continuous recording method. *Br. J. exp. Path.,* **55**, 1–12.

WILLOUGHBY, D A (1968). Effects of prostaglandins PGF$_{2\alpha}$ and PGE$_1$ on vascular permeability. *J Path. Bact*, **96**, 381–387.

ZACHARIAE, E. & ZACHARIAE, H (1965). The histamine content of human synovial membrane. A spectrofluorometric assay *Acta Rheum. Scand.,* **11**, 107–111.

ZWEIFACH, B W (1964). Microcirculatory aspects of tissue injury. *Ann. N.Y. Acad. Sci.,* **116**, 831–838.

(Received September 2, 1975)

Br. J. Pharmac. (1976), **56**, 317–322

THE EFFECTS OF PROSTAGLANDINS E_1, E_2 AND $F_{2\alpha}$ ON THE CUTANEOUS VASCULATURE OF THE RAT

LORIS A. CHAHL & R J. LADD
Department of Physiology, University of Queensland, St Lucia, Qld 4067, Australia

1 The responses of the rat cutaneous vasculature to prostaglandins E_1, E_2 and $F_{2\alpha}$ have been investigated by a photomicrographic technique.

2 Prostaglandin E_1 produced transient arterial constriction which was blocked by local pretreatment of preparations with compound 48/80 or methysergide. It was concluded that prostaglandin E_1 produced vasoconstriction by release of 5-hydroxytryptamine (5-HT) from mast cells. The magnitude of the vasoconstrictor response appeared to be subject to seasonal variation.

3 Prostaglandin $F_{2\alpha}$ produced arterial constriction of longer duration which was not blocked by compound 48/80, methysergide or phenoxybenzamine.

4 Preparations pretreated with prostaglandin $F_{2\alpha}$ were found to be more sensitive to the venous constrictor effect, and less sensitive to the arterial constrictor effect, of noradrenaline.

5 Prostaglandin E_2 produced arterial constriction which was usually partially blocked by compound 48/80 and methysergide and it was concluded that a major component of the vasoconstrictor response to prostaglandin E_2 was of the E_1 type but some component was of the $F_{2\alpha}$ type.

Introduction

Prostaglandins E_1 and E_2 have been widely reported to produce vasodilatation in a number of vascular beds whereas prostaglandin $F_{2\alpha}$ has been found to produce variable responses (see Higgins & Braunwald, 1972; Muirhead, 1973; Nakano, 1973). Few studies have been made on the cutaneous vasculature of the rat. Since this species is so often used for tests of inflammatory responses a study of the effects of these three prostaglandins on rat cutaneous vasculature was undertaken.

Methods

Male rats derived from the Wistar strain weighing 100 to 180 g were anaesthetized with urethane (1250–1500 mg/kg, i.p.). A longitudinal incision was made in the skin of either the abdomen or the outer thigh. The skin was carefully separated from the underlying tissues and a flap with the inner surface exposed, was placed over a translucent, perspex table (1.5 cm diameter) on a microscope stage. The flap was gently anchored in place with threads and transilluminated with a tungsten-halogen light source. Magnification was 19×. Photomicrographs of the silhouettes were taken at timed intervals with an Olympus PM-6 camera. Although the field was too deep to be all in focus, semiquantitative estimates of changes in diameter of the blood vessels were possible. The skin flap was kept moist with regular applications of Tyrode solution (composition g/l: NaCl 8.0, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, NaH_2PO_4 0.05, $NaHCO_3$ 1.0, glucose 1.0) at 37°C. Drugs were diluted in Tyrode at 37°C immediately before use and were dropped directly on to the tissue from a Pasteur pipette. Photomicrographs were usually taken at 15 s intervals for the first minute after application of a drug and thereafter at 1 min intervals until the response was maximal (2 to 4 minutes). The drug was then washed from the tissue with several changes of warm Tyrode solution. Two consistent responses to active drugs were always obtained before the addition of antagonists.

Drugs

The following drugs were used: compound 48/80 (Wellcome), histamine diphosphate (Sigma), 5-hydroxytryptamine creatinine sulphate (Sigma), methysergide hydrogen maleate (Sandoz), (–)-noradrenaline bitartrate (l-arterenol bitartrate, Sigma), phenoxybenzamine hydrochloride (Smith, Kline & French), prostaglandins E_1 (free acid), E_2 (free acid) and $F_{2\alpha}$ tromethamine salt (Upjohn). Concentrations given refer to weight of salt per ml of solution, except for prostaglandins E_1 and E_2 where concentrations refer to weight of free acid.

Results

Effects of prostaglandins on abdominal and outer thigh skin vasculature

Prostaglandin E_1 (10–200 ng/ml) produced transient constriction of the arteries of both abdominal skin and outer thigh skin, which usually was more marked on the vessels of the outer thigh skin. There was little effect on the veins. The constrictor effect appeared at about 45 s after application of prostaglandin E_1 and usually had disappeared by 2 minutes. It was often preceded by a brief, marked dilatation which was apparent within 15 seconds. Higher concentrations of prostaglandin E_1 (500 ng/ml to 10 µg/ml) did not produce constrictor effects (Figure 1 e–h); dilatation often occurred at these concentrations. There was some tachyphylaxis to the constrictor effect of prostaglandin E_1 but the responses could be repeated several times at 10 min intervals.

A seasonal variation in the constrictor response to prostaglandin E_1 was found, the response being much less during the summer months (October to April) than during the winter months (May to September). On many preparations tested during the summer months it produced only a vasodilator effect but vaso-

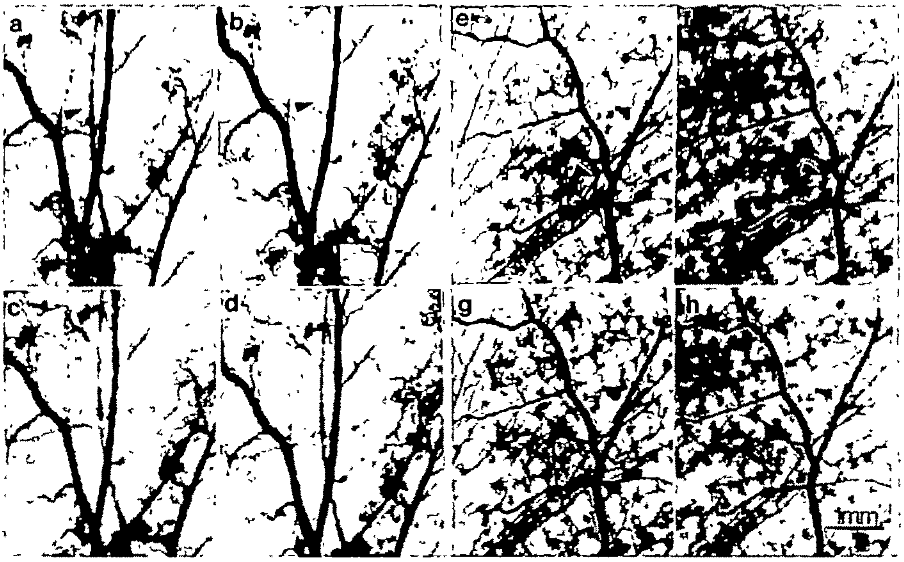


Figure 1 Effect of compound 48/80 on the response of rat cutaneous vasculature to prostaglandin E_1 (100 ng/ml) (outer thigh skin): (a) Control, (b) 2 min after application of prostaglandin E_1 (note arterial constriction at arrow), (c) control after two applications of 48/80 10 µg/ml; (d) 2 min after application of prostaglandin E_1 (note absence of arterial constriction) Effect of low and high concentrations of prostaglandin E_1 on rat cutaneous vasculature (outer thigh skin): (e) Control; (f) 2 min after application of prostaglandin E_1, 10 ng/ml (note arterial constriction at arrow); (g) control; (h) 2 min after application of prostaglandin E_1, 10 µg/ml (note absence of arterial constriction).

Table 1 Vasoconstrictor responses to prostaglandins in three seasons

	First winter		*Summer*		*Second winter*	
	Tested	*Constricted*	*Tested*	*Constricted*	*Tested*	*Constricted*
Prostaglandin E_1						
Abdomen	6	4	11	2 (slight)	3	3
Thigh	6	6	27	7 (slight)	6	5
Prostaglandin E_2						
Abdomen	–	–	–	–	5	5
Thigh	–	–	4	0	7	7
Prostaglandin $F_{2\alpha}$						
Abdomen	–	–	3	3	3	3
Thigh	–	–	4	4	5	5

Abdomen—abdominal skin, Thigh—outer thigh skin. A dash indicates that no tests were made constrictor effects were observed during two winter periods (Table 1).

Prostaglandin E_2 (100–200 ng/ml) also produced a constrictor effect on arteries. However on 4 preparations where both prostaglandin E_1 and E_2 were tested, it was found that the constrictor effect of prostaglandin E_2 was longer lasting and more generalized over the whole field than was that of E_1. The constrictor effect of prostaglandin E_2 was also preceded by a phase of dilatation.

Prostaglandin $F_{2\alpha}$ (50 ng/ml to 1 μg/ml) produced marked constriction of arteries, sometimes preceded by dilatation. The constrictor effect developed more slowly than that of prostaglandin E_1 and appeared between 1 and 2 min after addition of the drug. The constrictor response to prostaglandin $F_{2\alpha}$ differed from that to E_1 in the more generalized nature of the response (Figure 3b). In some preparations slight venous constriction also occurred. The preparations exhibited marked tachyphylaxis to the constrictor effect and to obtain reproducible responses additions of prostaglandin $F_{2\alpha}$ were made at 30 min intervals. There was no evidence of seasonal variation in the constrictor response to prostaglandin $F_{2\alpha}$ (Table 1).

Effect of compound 48/80 on responses to prostaglandins

Addition of compound 48/80 (10 or 100 μg/ml) to the preparations produced marked constriction of the arteries with little effect on the veins (Figure 2a,b). Very low concentrations of 5-HT (1 ng/ml) produced vasodilatation. Higher concentrations of 5-HT (10 to 100 ng/ml), but not histamine (100 ng/ml to 10 μg/ml), produced marked constriction of the arteries (Figure 2e–h). It therefore seemed likely that the effects observed with compound 48/80 were due to 5-HT released from mast cells. In order to deplete the mast cells of amines, two or three additions of compound 48/80 were made until there was no constrictor response (Figure 2c,d). The response of the preparations to 5-HT was not affected by pretreatment with compound 48/80.

The constrictor response to prostaglandin E_1 was completely abolished by pretreatment with compound 48/80 on all preparations tested (4 thigh skin and 2 abdominal skin preparations) (Figure 1a–d). The effect of prostaglandin E_2 was partially blocked on 2 thigh skin and 1 abdominal skin preparation and completely blocked on 1 thigh skin and 1 abdominal skin preparation. The action of prostaglandin $F_{2\alpha}$ was not blocked on any of the preparations tested (3 thigh skin and 3 abdominal skin preparations). The dilator responses to prostaglandins E_1 and E_2 did not appear to be blocked by pretreatment with compound 48/80.

Effect of methysergide on responses to prostaglandins

Methysergide (1 μg/ml) dripped on to the preparations for 5 min produced complete block of the constrictor

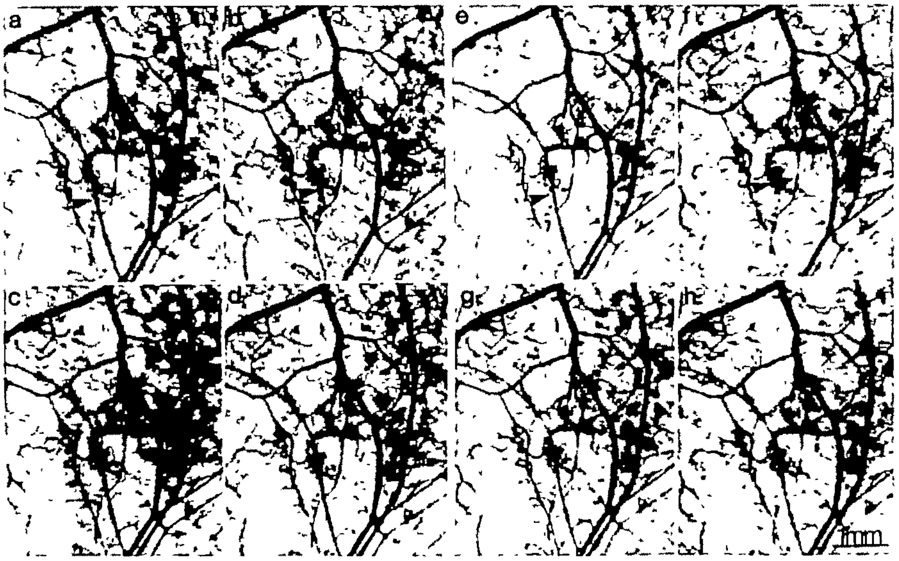


Figure 2 Effect of compound 48/80, 5-hydroxytryptamine (5-HT) and histamine on rat cutaneous vasculature (outer thigh skin) Effect of first and second application of compound 48/80 (10 μg/ml): (a) control; (b) 2 min after first application of compound 48/80 (note complete closure of both arteries at arrows); (c) control, (d) 2 min after second application of same amount of compound 48/80 (note absence of arterial constriction). Effect of 5-HT (10 ng/ml) (e) control, (f) 1 min after application of 5-HT (note almost complete closure of one artery at arrow) Effect of histamine (10 μg/ml): (g) control; (h) 2 min after application of histamine (note absence of arterial constriction).

response to 5-HT (100 ng/ml). The responses to the prostaglandins were tested before and after treatment of the preparations with methysergide (1 μg/ml) for 5 minutes. The constrictor response to prostaglandin E_1 was completely blocked by methysergide (5 thigh skin preparations), that to prostaglandin E_2 was partially blocked (4 thigh skin and 3 abdominal skin preparations), and that to prostaglandin $F_{2\alpha}$ was unaffected (2 thigh skin and 1 abdominal skin preparation).

Effect of phenoxybenzamine on the response to prostaglandin $F_{2\alpha}$

Phenoxybenzamine (1 μg/ml) dripped on to the preparations for 5–15 min produced complete block of the constrictor response to noradrenaline (100–200 ng/ml) (Figure 3c–d, g–h). On the 2 abdominal skin preparations tested the constrictor response to prostaglandin $F_{2\alpha}$ was not blocked by phenoxybenzamine and may even have been enhanced (Figure 3a–b, e–f). On these preparations it was also noted that noradrenaline produced more marked constriction of veins than arteries whereas prostaglandin $F_{2\alpha}$ produced marked arterial constriction (Figure 3). In both experiments noradrenaline was tested after application of prostaglandin $F_{2\alpha}$ to the preparations.

Discussion

The rat cutaneous vascular preparation used in these experiments was very reactive to topically applied drugs and, in contrast to the rat mesenteric vascular preparation studied by Furness & Marshall (1974) was capable of responding to drugs with virtually complete closure of the arteries (Figure 2).

The results presented in this paper have shown that prostaglandins E_1, E_2 and $F_{2\alpha}$ produce constrictor responses of the rat cutaneous vasculature. However the nature of the responses and the mechanisms by which they were produced appeared to differ. Prostaglandin E_1 produced arterial constriction of relatively short duration which was blocked by compound 48/80 and by methysergide. Since it is known that rat mast cells contain 5-HT as well as histamine (Benditt, Wong, Arase & Roeper, 1955) and 5-HT and compound 48/80 both produced arterial constriction of this preparation, while histamine did not, it would appear that the constrictor effect of prostaglandin E_1 was due to 5-HT released from mast cells. In other species where histamine is the only amine present in mast cells, and where histamine produces vasodilatation, a vasodilatator response only to prostaglandin E_1 would be expected. Vasodilatation to prostaglandin E_1 has in fact been observed on most vascular beds where it has been tested (see Higgins &

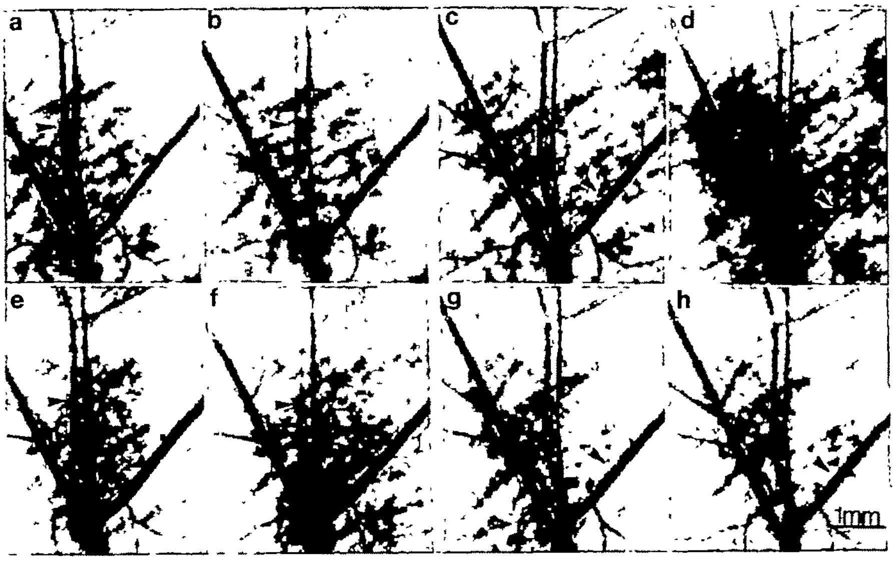


Figure 3 Effect of phenoxybenzamine (1 μg/ml) on the responses of rat cutaneous vasculature to prostaglandin $F_{2\alpha}$ (200 ng/ml) and noradrenaline (100 ng/ml): (a) control; (b) 2 min after application of prostaglandin $F_{2\alpha}$ (note arterial constriction at arrow) (c) control; (d) 2 min after application of noradrenaline (note venous constriction at arrow); (e) control after application of phenoxybenzamine for 15 min; (f) 2 min after application of prostaglandin $F_{2\alpha}$ (note arterial constriction still present); (g) control after application of phenoxybenzamine; (h) 2 min after application of noradrenaline (note absence of venous constriction at arrow).

Braunwald, 1972; Muirhead, 1973; Nakano, 1973), although constriction of dog nasal blood vessels (Stovall & Jackson, 1967) and rat mesocaecum metarterioles (Viguera & Sunahara, 1969) has been reported. Our results indicate that dose might be an important factor in determining the response observed with prostaglandin E_1 since higher concentrations of the drug (500 ng/ml to 10 µg/ml) did not produce vasoconstriction, presumably because some other action masked the constrictor response. The mechanisms of the dilator responses to prostaglandin E_1 observed at very low doses and very high doses were not investigated. However low doses of 5-HT produced vasodilatation and it is possible that the vasodilatation observed with low doses of prostaglandin E_1 might also be due to released 5-HT.

The seasonal variation observed in the response to prostaglandin E_1 might reflect some variation in the storage or release of 5-HT from rat mast cells. 5-HT levels in the central nervous system have been found to fall in rats in a hot environment and to rise in rats in a cold environment (Corrodi, Fuxe & Hökfelt, 1967), but it is not known whether there is any temperature or seasonal variation in the levels of 5-HT in rat mast cells. Prostaglandin E_1 has been shown to produce oedema by causing release of amines from mast cells (Crunkhorn & Willis, 1971) but Freeman & West (1972) found marked differences in the skin response to prostaglandin E_1 between rats from different breeding colonies. It is possible that seasonal variation might have been responsible for some of the differences observed by these workers. The greater sensitivity of the outer thigh skin vessels to the constrictor action of prostaglandin E_1 which was often observed, might reflect a larger number of mast cells in the skin of this area than in abdominal skin, or greater storage of 5-HT in mast cells of the outer thigh skin. Parratt & West (1957) found that the 5-HT levels in the skin of the groin of the rat were higher than those in the abdominal skin.

A major component of the constrictor response to prostaglandin E_2 also appeared to be due to released 5-HT but there was some component of the response which was not blocked by compound 48/80 or methysergide, and which, in time course and appearance, resembled the constrictor response to prostaglandin $F_{2\alpha}$. The constrictor response to prostaglandin $F_{2\alpha}$ was of longer duration, was more generalized over the whole lengths of the arteries, sometimes involved the small veins, and was more markedly tachyphylactic than that to prostaglandin E_1.

The site and mechanism of action of prostaglandin $F_{2\alpha}$ on the peripheral vasculature is controversial (see Muirhead, 1973; Nakano, 1973). Results from our experiments suggest that prostaglandin $F_{2\alpha}$ has two actions. Pretreatment with this prostaglandin appeared to enhance the venous constrictor effect of noradrenaline but antagonize the arterial constrictor effect. At the concentrations used in our experiments prostaglandin $F_{2\alpha}$ also produced a vasoconstrictor effect, predominantly on arteries, which did not involve 5-HT or adrenergic mechanisms since it was not blocked by methysergide or phenoxybenzamine. It would seem therefore that the observed response to prostaglandin $F_{2\alpha}$ might vary with both the dose and the level of the natural adrenergic tone. This might explain the apparently conflicting observations in the literature. In the presence of high adrenergic tone, prostaglandin $F_{2\alpha}$ might produce venoconstriction. A venopressor action, which was dependent on sympathetic innervation, has indeed been observed in the perfused dog limb (Ducharme, Weeks & Montgomery, 1968). However, an arterial constrictor effect independent of sympathetic innervation has also been described in the dog (Nakano, Perry & Denton, 1968). The decrease in sensitivity to the arterial constrictor action of noradrenaline might explain the lack of potentiation of noradrenaline on the perfused dog hind paw preparation (Kadowitz, Sweet & Brody, 1971; 1972), and the vasodilator action of prostaglandin $F_{2\alpha}$ in the cat (Änggård & Bergström, 1963). Further experiments are required to clarify the interaction of prostaglandins with adrenergic mechanisms on the cutaneous vasculature.

We wish to thank Dr J.E. Pike of the Upjohn Company, Kalamazoo, Michigan for generous samples of prostaglandins and Dr P. Isaac of Sandoz Australia Pty. Ltd. for a gift of methysergide. Technical assistance by Miss Anne Schafferius is gratefully acknowledged. The work was supported by a University Research Grant.

References

ÄNGGÅRD, E & BERGSTRÖM, S. (1963). Biological effects of an unsaturated trihydroxy acid ($PGF_{2\alpha}$) from normal swine lung. *Acta physiol. scand.*, **58**, 1–12.

BENDITT E.P., WONG, R L, ARASE, M. & ROEPER, E (1955). 5-hydroxytryptamine in mast cells. *Proc. Soc. exp. Biol. Med.*, **90**, 303–304.

CORRODI, H., FUXE, K & HÖKFELT, T. (1967). A possible role played by central monoamine neurones in thermoregulation. *Acta. physiol. scand.*, **71**, 224–232.

CRUNKHORN, P. & WILLIS, A.L (1971). Cutaneous reactions to intradermal prostaglandins. *Br. J. Pharmac.*, **41**, 49–56.

DUCHARME, D.W., WEEKS, J.R & MONTGOMERY, R.G. (1968). Studies on the mechanism of the hypertensive effect of prostaglandin $F_{2\alpha}$. *J. Pharmac. exp. Ther.*, **160**, 1–10.

FREEMAN, P.C. & WEST, G.B. (1972). Skin reactions to prostaglandins. *J. Pharm. Pharmac.*, **24**, 407–408.

Br. J. Pharmac. (1976), 56, 323–330

A COMPARISON OF γ-AMINOBUTYRIC ACID AND THE SEMI-RIGID ANALOGUES 4-AMINOTETROLIC ACID, 4-AMINOCROTONIC ACID AND IMIDAZOLE-4-ACETIC ACID ON THE ISOLATED SUPERIOR CERVICAL GANGLION OF THE RAT

N.G. BOWERY[1]
Department of Pharmacology, The School of Pharmacy,
University of London, 29/39 Brunswick Square, London WC1N 1AX
G.P. JONES
William Ramsay, Ralph Forster and Christopher Ingold Laboratories,
University College London, Gower Street, London WC1E 6BT

1 The rat superior cervical ganglion possesses receptors for γ-aminobutyric acid (GABA). This can be demonstrated *in vitro* by recording the changes in ganglionic surface potential which occur after the addition of GABA to the bathing solution.

2 The actions of three conformationally-restricted analogues of GABA namely 4-aminotetrolic acid (4-ATA), *trans* 4-aminocrotonic acid (4-ACA) and imidazole-4-acetic acid (IAA) have been examined for activity at this peripheral receptor.

3 All three analogues depolarized the ganglion in a manner similar to GABA. Their actions were transient and were 'occluded' by GABA; also the log dose-response curve in each case was parallel to that of GABA. Molar potencies relative to GABA (=1) were 4-ACA=1.48, IAA=0.100, 4-ATA=0.0028.

4 The action of each analogue could be blocked by the GABA antagonists bicuculline and tetramethylenedisulphotetramine at doses which had relatively little effect on responses to the cholinomimetic carbachol.

5 4-ACA and IAA (1 mM) significantly reduced the ganglionic accumulation of [^{3}H]-GABA (0.2 μM) by 88% and 58% respectively whereas 4-ATA (1 mM), caused no significant reduction in [^{3}H]-GABA accumulation.

Introduction

4-Aminotetrolic acid (4-ATA), *trans*-4-aminocrotonic acid (4-ACA) and imidazole-4-acetic acid (IAA) (Figure 1) can be considered as conformationally-restricted analogues of γ-aminobutyric acid (GABA) the putative central inhibitory transmitter (see Curtis & Johnston, 1974). All three analogues show considerable GABA-like activity on neurones in the mammalian central nervous system: iontophoretic application produces a marked inhibition in neuronal cell firing, and this can be antagonized by bicuculline, N-methyl bicuculline or picrotoxin. Furthermore they are resistant to the action of strychnine, suggesting that their effect resembles that of GABA rather than glycine (Krnjević & Phillis, 1963; Phillis, Tebecis & York, 1968; Beart, Curtis & Johnston, 1971; Curtis, Duggan, Felix & Johnston, 1971; Curtis, Duggan, Felix, Johnston & McLennan, 1971; Godfraind, Krnjević, Maretić & Pumain, 1973; Haas, Anderson & Hösli, 1973; Dray, 1975; Johnston, Curtis, Beart, Game, McCulloch & Twitchin, 1975).

Receptors for GABA in the mammal are not confined to the central nervous system: depolarization of the cat superior cervical, vagal sensory and dorsal root ganglia *in vivo* (de Groat, 1970, 1972; de Groat, Lalley & Saum, 1972) and the rat superior cervical ganglion *in vitro* (Bowery & Brown, 1974), and release of catecholamines from isolated bovine adrenals (Sangiah, Borowitz & Yim, 1974) can be evoked by GABA. The chemical specificity of the receptors for GABA in the rat superior cervical ganglion (Bowery & Brown, 1974) together with the evidence that GABA depolarizes the ganglionic neurones by

---

[1] Present address: Department of Pharmacology, St. Thomas's Hospital Medical School, London SE1 7EH.

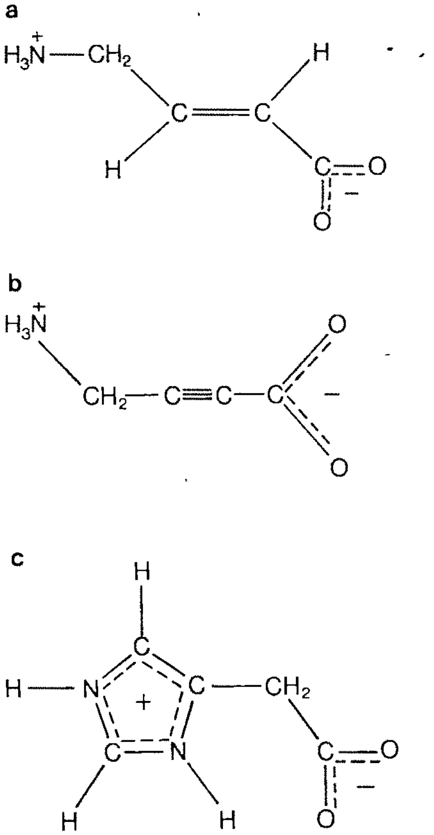


Figure 1 Structures of (a) 4-aminocrotonic acid, (b) 4-aminotetrolic acid and (c) imidazole-4-acetic acid

increasing Cl^- conductance, (Adams & Brown, 1975) indicates a close resemblance between GABA receptors on sympathetic ganglion cells and those on central neurones. These receptors have certain advantages for experimental study, for instance, drugs can be applied in known concentrations which facilitates potency measurements. In the present experiments we have compared quantitatively the effects of 4-ATA, 4-ACA, IAA and GABA on the rat superior cervical ganglion the results of which may prove helpful in interpreting structural requirements for activation of the GABA receptor.

Methods

Surface depolarization of rat isolated superior cervical ganglia was measured by the superfusion technique described elsewhere (Bowery, Brown & Collins, 1975; Brown & Marsh, 1975). Ganglia were dissected from Wistar rats anaesthetized with urethane (1.5 g/kg) and were desheathed before use.

The effect of the analogues on GABA accumulation by isolated ganglia was determined by incubating ganglia in Krebs solution (1 ml/ganglion) containing 0.2 μM [^{3}H]-GABA ([^{3}H-2-3]-γ-aminobutyric acid, 10 Ci/mmol, New England Nuclear) in the absence or presence of a 1 mM concentration of the analogues. Incubations were continued for 30 min at 25 °C in the presence of 10 μM amino-oxyacetic acid to prevent GABA metabolism (Walsh, Bowery, Brown & Clark, 1974). The amount of [^{3}H]-GABA accumulated by individual ganglia was determined by scintillation counting (see Bowery & Brown, 1972a) and expressed as a tissue : medium ratio (d min^{-1} mg^{-1} ganglion : d min^{-1} μl^{-1} bath fluid).

Imidazoleacetic acid was obtained from Sigma Ltd. 4-Aminotetrolic acid and *trans*-4-aminocrotonic acid were prepared by the methods of Beart & Johnston (1972) and Musashi (1954) respectively and subsequently re-crystallized.

(+)-Bicuculline methochloride (methylbicuculline) and tetramethylenedisulphotetramine (TETS) were synthesized by J.F. Collins (Dept. of Chemistry, Sir John Cass School of Science) according to the methods of Johnston, Beart, Curtis, Game, McCulloch & MacLachlan (1972) and Hecht & Heneka (1949).

Results

Depolarization

Superfusion of the rat isolated superior cervical ganglion with solutions containing IAA, 4-ATA or 4-ACA produced a dose-dependent depolarization of the ganglion (Figure 2). The threshold concentration in each case was 5–20 μM (IAA), 300–1000 μM (4-ATA) and 1–3 μM (4-ACA). The depolarizations produced by these compounds showed the following features characteristic of those produced by GABA (Figures 3 and 4).

Firstly, their effects were transient; the peak depolarization occurred within 30 s and then declined in spite of continued application. This contrasted with the depolarization produced by carbachol which was slower in onset and was sustained for at least 4 min (see Bowery & Brown, 1974). Secondly, the log dose-response curves to IAA, 4-ATA and 4-ACA paralleled that to GABA (Figure 2). However, the maximum response to IAA was always less than that produced by GABA, and a maximum response to 4-ATA was never obtained due to its low potency. Thirdly, during prolonged superfusion with GABA, the addition of IAA, 4-ATA and 4-ACA did not depolarize the ganglion whereas carbachol still produced a response. This 'occlusion' phenomenon in the ganglion has previously been described for other GABA analogues (Bowery & Brown, 1972b, 1974).

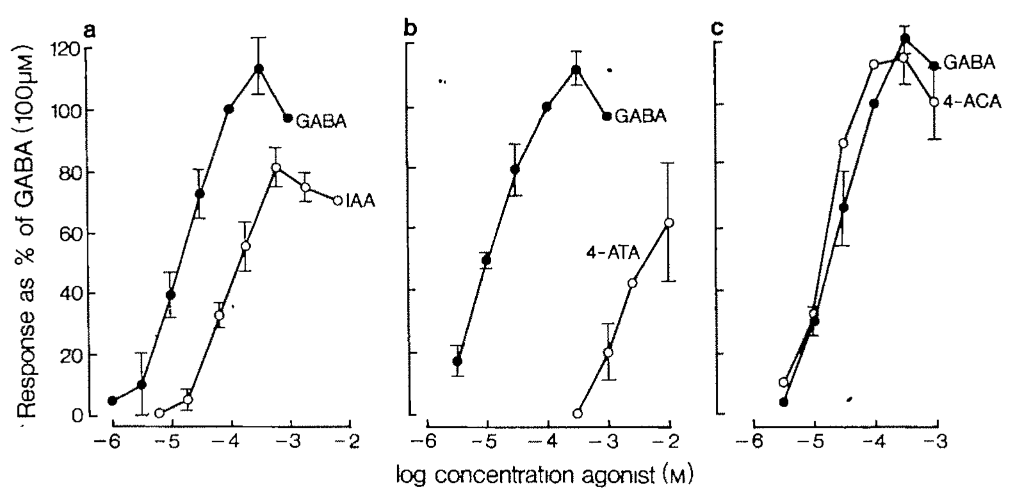


Figure 2 Log dose-response curves for (a) imidazole-4-acetic acid (IAA), (b) 4-aminotetrolic acid (4-ATA), (c) *trans*-4-aminocrotonic acid (4-ACA) and γ-aminobutyric acid (GABA)-induced (●) depolarizations of the rat isolated superior cervical ganglion Compounds were applied for 1 min at 15 min intervals. Data were obtained from 3 or 4 separate experiments for each compound in which GABA was compared with one or more of the analogues Each point represents the mean plus or minus standard errors (vertical bars) where $n > 3$. Abscissae: log molar agonist concentration Ordinates mV surface depolarization plotted as % of response to GABA 100 μM in the same experiment

Fourthly, depolarization by the analogues was antagonized by bicuculline or methylbicuculline (Figure 3) and by tetramethylenedisulphotetramine (TETS) (Figure 4) (see Bowery *et al.*, 1975) at concentrations which produced less than 15% reduction of responses to carbachol. It may be noted that the responses to carbachol shown in Figures 3 and 4 were approximately 20% of the maximum response to this substance in these experiments, whereas the responses to GABA of comparable magnitude were approximately 75% of the maximum response to GABA. Moreover at equal levels of depolarization, the

Table 1 The effect of 1 mM concentrations of 4-amino-tetrolic acid (4-ATA), imidazole-4-acetic acid (IAA), *trans*-4-aminocrotonic acid (4-ACA) and γ-aminobutyric acid (GABA) on the uptake of [^{3}H]-GABA by individual ganglia.

	Mean tissue· medium ratio	*% inhibition of [^{3}H]-GABA uptake*	*Depolarization molar potency of analogue*
Control	13.59 ± 0.94 ($n=5$)		
+ 4-ATA (1 mM)	12.18 ± 1.80 ($n=5$)	Not significant	0.0028
+ IAA (1 mM)	*5 76 ± 0.59 ($n=4$)	58%	0.100
+ 4-ACA (1 mM)	*1.58 ± 0.08 ($n=5$)	88%	1.48
+ GABA (1 mM)	*0.64 ± 0 43 ($n=3$)	95%	1

*Value significantly different from control ($P<0\ 001$).

Ganglia were incubated for 30 min at 25°C in the presence of [^{3}H]-GABA 0 2 μM (plus amino-oxyacetic acid 10 μM). The ganglia were *not* pre-incubated with the analogues. Mean tissue· medium ratios are shown together with the standard error of the mean in each case. The number of ganglia (n) used for each determination is shown in brackets. Molar potencies for depolarization (see text) are included for comparison.

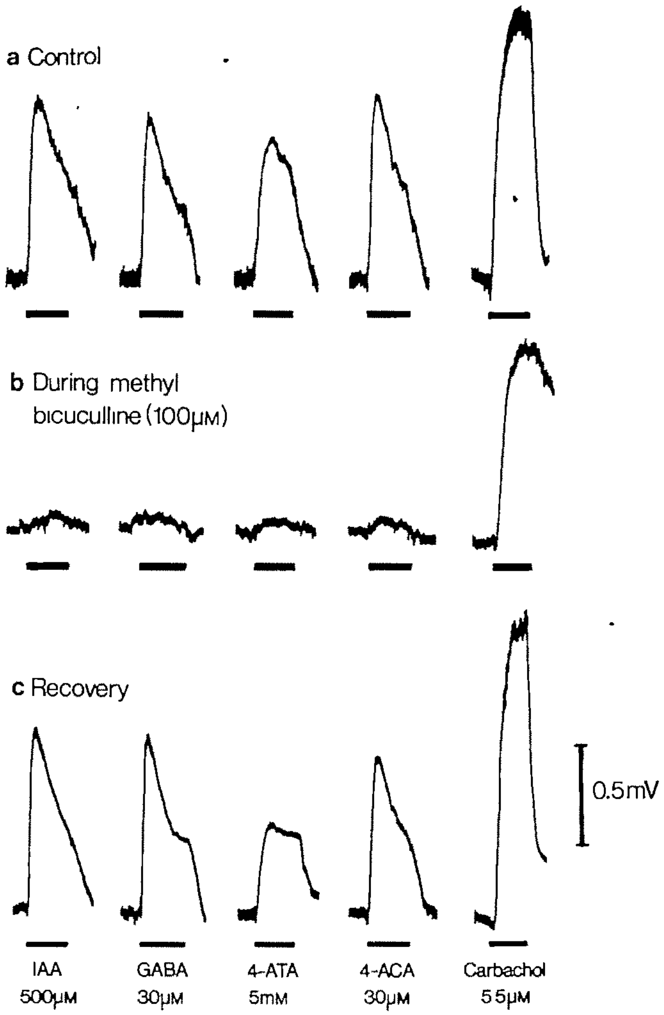


Figure 3 The effect of methyl bicuculline (100 μM) on ganglionic depolarizations produced by imidazole-4-acetic acid (IAA, 500 μM), γ-aminobutyric acid (GABA, 30 μM), 4-aminotetrolic acid (4-ATA, 5 mM), *trans*-4-aminocrotonic acid (4-ACA, 30 μM) and carbachol (5.5 μM). The results were obtained from a single ganglion. Compounds were applied for 1 min (indicated by horizontal bars) at 15 min intervals Calibration bar 0.5 mV. (a) Was obtained before, (b) during, and (c) 30 min after the application of methyl bicuculline (100 μM). Note the selective antagonism of GABA and its analogues

conductance change produced by carbachol is much less than that produced by GABA (Adams & Brown, 1975), even though the maximal conductance increase that can be induced by carbachol is far in excess of the maximum induced by GABA. This would tend to make the response to carbachol more sensitive to 'non-specific' reduction than that to GABA.

The molar potencies of the analogues compared to GABA (= 1) determined from the data in Figure 2 by linear regression analysis around the 50% level on the ordinates were IAA = 0.100, 4-ATA = 0.0028 and 4-ACA = 1.48.

Transport of [^{3}H]-γ-aminobutyric acid

Exogenous GABA is accumulated into ganglionic glial cells by a high-affinity carrier-mediated transport process (Bowery & Brown, 1972a; Young, Brown, Kelly & Schon, 1973). Ganglia incubated in low concentrations (< 100 μM) of [^{3}H]-GABA accumulate this amino acid against a concentration gradient. The additional presence of 1 mM IAA or 4-ACA in the incubation medium significantly decreased the uptake of [^{3}H]-GABA during 30 min incubation (Table 1). However, 4-ATA (1 mM) did not significantly reduce

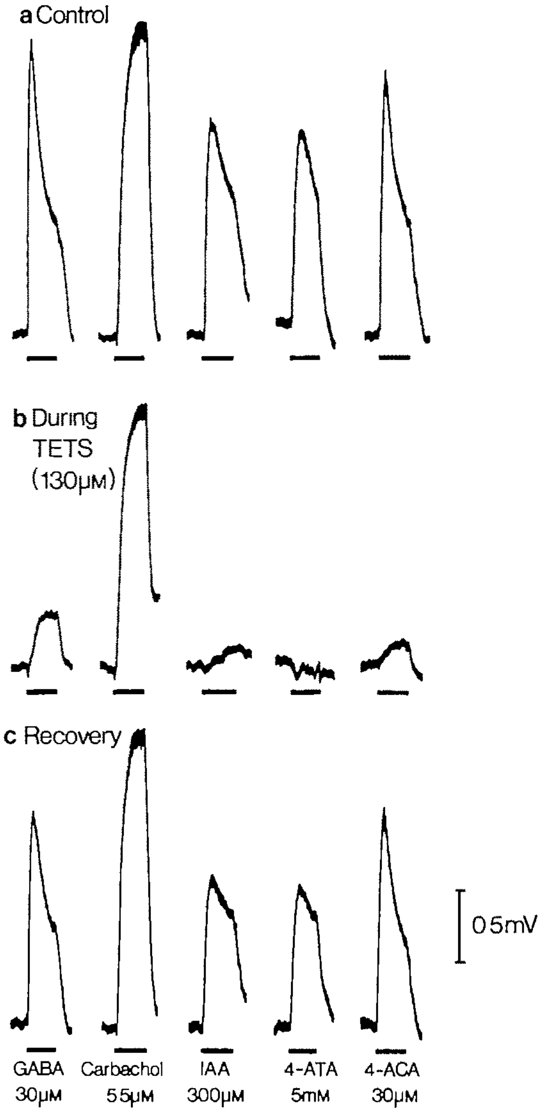


Figure 4 Details as for Figure 3 except that tetramethylenedisulphotetramine (TETS, 130 µM) was used in place of methyl bicuculline and the imidazole-4-acetic acid (IAA) concentration was 300 µM TETS was dissolved in 0.3% acetone (see Bowery, Brown & Collins, 1976) The results were obtained in a different ganglion from that shown in Figure 3.

GABA accumulation. The order of potency in inhibiting the uptake of GABA was 4-ACA (88%) > IAA (58%) > 4-ATA (10%).

Discussion

The results show that 4-ACA, IAA and 4-ATA all produced a surface depolarization of the isolated superior cervical ganglion in a manner resembling that of GABA. An action at 'GABA receptors' in this tissue (see Bowery & Brown, 1974) is supported in all three cases by (i) the transient nature of responses to the analogues (ii) their parallel log dose-response curves (iii) their 'occlusion' by GABA and (iv) their antagonism by methyl bicuculline and tetramethylenedisulphotetramine. Such an action accords with previous findings in the mammalian central nervous system (see introductory section).

Trans-4-ACA was equipotent with GABA at the ganglionic receptors, a result which compares with that described by Johnston *et al.* (1975). Iontophoretic application of *trans*-4-ACA on to spinal interneurones inhibited cell firing to approximately the same extent as GABA. In contrast, however, the potencies of 4-ATA and IAA in the ganglion (approximately 1/400 and 1/10 of GABA respectively) appear to differ from those estimated at receptors within the central nervous system. Beart *et al.* (1971) indicated that '4-ATA was between one half and one fifth as active as GABA' in depressing spinal interneurones, and other workers have shown that IAA appears to be as active as GABA in decreasing neuronal cell firing in the cerebellum, spinal cord (Curtis *et al.*, 1971) and brain stem (Haas *et al.*, 1973) and about half as active as GABA on cortical neurones (Godfraind *et al.*, 1973). These values are based on electrophoretic currents required to expel sufficient of the drugs to depress cell firing to a similar extent (often maximally); but, even using an alternative means of quantitation (see Hill & Simmonds, 1973) iontophoretically-applied IAA appears to be as potent as GABA in the rat brain stem (Dray, 1975). Nevertheless, a direct comparison between values obtained for IAA and 4-ATA applied iontophoretically in the central nervous system and in the ganglion *in vitro* is difficult in view of the difficulty in expressing potency levels in terms of drug concentration in the former. However, the recent findings of Barker, Nicoll & Padjen (1975) using frog isolated spinal cord indicate that IAA has 2.5 times the potency of GABA in depolarizing primary afferent terminals; and Zukin, Young & Snyder (1974) have shown that IAA is almost as potent as GABA in binding to 'GABA receptors' in synaptic membrane fractions of the rat central nervous system.

Thus, the apparent difference in potencies between IAA and 4-ATA in the central nervous system and ganglion may well be 'real'. Certain factors may contribute to this difference. For example, in brain tissue the accumulation of GABA is greater (cf. Iversen & Neal, 1968 with Bowery & Brown, 1972a) possibly due to the presence of a neuronal uptake component not present in the ganglion (Young *et al.*, 1973). Since both 4-ATA and IAA are poor inhibitors of GABA uptake in cortical or spinal cord slices and brain synaptosomes (Beart, Johnston & Uhr, 1972;

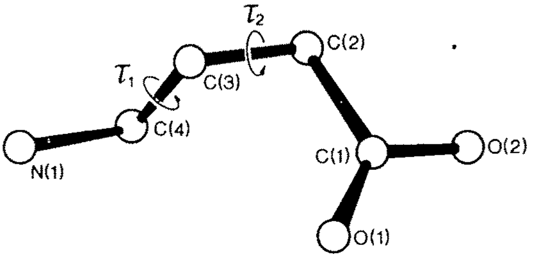


Figure 5 The solid state conformation of γ-aminobutyric acid (GABA) illustrating the torsion angles τ_1 and τ_2 which are used to describe the conformations of the rigid analogues of GABA.

Balcar & Johnston, 1973; Zukin *et al.*, 1974) they might also be accumulated to a much lesser extent thereby increasing their effective concentrations in the region of the receptors.

It is unlikely that the greater potency of IAA or 4-ATA in the central nervous system relates to an additional action at glycine receptors (ganglia are devoid of receptors for glycine, Bowery & Brown, 1974) since strychnine is ineffective as an antagonist of the actions of either 4-ATA or IAA when applied at iontophoretic currents capable of antagonizing glycine (Beart *et al.*, 1971; Curtis *et al.*, 1971; Haas *et al.*, 1973). The insensitivity of IAA to strychnine in the frog spinal cord is confused by the evidence that glycine-evoked primary afferent depolarization is also insensitive to the action of strychnine (Barker *et al.*, 1975).

The molecular conformations of 4-ATA, *trans*-4-ACA and IAA can be illustrated by consideration of the torsion angles (Klyne & Prelog, 1960) τ_1 (N(1)–C(4)–C(3)–C(2)) and τ_2 (C(4)–C(3)–C(2)–C(1)) depicted on the GABA molecule shown in Figure 5. In 4-ACA, τ_2 is fixed at ~180° with free rotation occurring about the bond defined by τ_1. 4-ATA is linear because of the stereochemical requirements of the triple bond although free rotation is allowed about C(3)–C(4). IAA has τ_1 fixed at ~180°. In each of these molecules the separation of the negatively charged centre and the positively charged nitrogen atom of 5.8 ± 0.2 Å suggested by Kier, George & Höltje (1974) for activity at the GABA receptor is theoretically possible. X-ray crystal structure analyses on these compounds (Jones & Pauling, 1975, 1976a,b) show that in the solid state this is in fact the case (see Table 2).

Unlike the primary amine groups of GABA, 4-ATA and 4-ACA, IAA has a secondary amine functionality which is fixed in a planar configuration by the requirements of the imidazole ring, with only one hydrogen atom available for hydrogen bonding. If hydrogen bonding is important in the binding of the amine centre to the receptor then IAA is likely to be less effective than GABA. The fixed planar configuration appears to be important since N-methyl GABA, where the nitrogen atom is allowed to rotate freely about the N(1)–C(4) bond, has negligible activity both in the central nervous system (Curtis & Watkins, 1960) and in the ganglion (Bowery & Brown, 1974).

It has been suggested by Beart *et al.* (1972) that in the case of inhibition of GABA uptake by 4-ATA in rat brain slices the stronger acidity of 4-ATA may account for its weaker inhibition when compared with other structural analogues of GABA. This may also be an important factor when considering binding at the receptor. The low pK_1 (COO^-) value of 4-ATA (1.08) suggests a stronger ionic binding or hydrogen bonding capability of the carboxylate end of the molecule which may lead to an adverse distortion of the receptor conformation. Conversely, the low pK_2 (NH_3^+) value for IAA (7.46) suggests that the nitrogen centre may be less effective than in GABA (pK_2 10.71) for binding to the receptor. 4-ACA has similar pK_1 and pK_2 values to GABA. The differences in the action of 4-ATA and IAA in the central nervous system and in the ganglion may therefore illustrate differences in charge requirements of the substrates for these two systems.

The authors wish to thank Dr J.F. Collins for the preparations of N-methyl bicuculline and tetramethylene-disulphotetramine.

Table 2 Intramolecular N .... O distances (Å) for the restricted γ-aminobutyric acid (GABA) analogues

	N. . O[1]	*N. . . O[2]*	*Reference*
GABA	5.612 (±.006)	4.238 (± 007)	Tomita, Higashi & Fujiwara (1973)
4-ACA	5.657 (±.003)	5.052 (± 003)	Jones & Pauling (1975)
	5.098 (±.003)	5.130 (± 003)	
4-ATA	5 699 (± 004)	5.227 (±.004)	Jones & Pauling (1976a)
IAA	5 714 (±.004)	4.402 (±.004)	Jones & Pauling (1976b)

Estimated standard deviations in parentheses 4-ACA = *trans*-4-aminocrotonic acid; 4-ATA = 4-aminotetrolic acid, IAA = imidazole-4-acetic acid.

References

ADAMS, P.R & BROWN, D.A (1975). Actions of γ-aminobutyric acid on sympathetic ganglion cells. *J. Physiol., Lond.*, **250**, 85–120.

BALCAR, V.J. & JOHNSTON, G A.R. (1973). High affinity uptake of transmitters: studies on the uptake of L-aspartate, GABA, L-glutamate and glycine in cat spinal cord. *J Neurochem*, **20**, 529–539.

BARKER, J.L., NICOLL, R.A. & PADJEN, A (1975). Studies on convulsants in the isolated frog spinal cord. 1. Antagonism of amino acid responses. *J. Physiol., Lond.*, **245**, 521–536.

BEART, P M, CURTIS, D.R., JOHNSTON, G.A.R (1971). 4-Aminotetrolic acid: new conformational-restricted analogue of γ-aminobutyric acid. *Nature, New Biol.*, **234**, 80–81.

BEART, P.M & JOHNSTON, G.A R (1972). Acetylene analogues of γ-aminobutyric acid. *Aust. J. Chem.*, **25**, 1359–1361.

BEART, P M, JOHNSTON, G.A.R. & UHR, M L. (1972). Competitive inhibition of GABA uptake in rat brain slices by some GABA analogues of restricted conformation. *J. Neurochem.*, **19**, 1855–1861.

BOWERY, N G & BROWN, D.A. (1972a). γ-Aminobutyric acid uptake by sympathetic ganglia. *Nature, New Biol.*, **238**, 89–91.

BOWERY, N.G & BROWN, D A. (1972b). γ-Aminobutyrylcholine; actions on GABA and acetylcholine receptors. *J. Pharm Pharmac.*, **24**, 663–666.

BOWERY, N.G. & BROWN, D A (1974). Depolarizing actions of γ-aminobutyric acid and related compounds on rat superior cervical ganglia in vitro. *Br. J. Pharmac.*, **50**, 205–218.

BOWERY, N.G., BROWN, D.A. & COLLINS, J F (1975). Tetramethylenedisulphotetramine: an inhibitor of γ-aminobutyric acid induced depolarization of the isolated superior cervical ganglia of the rat. *Br. J. Pharmac.*, **53**, 422–424.

BROWN, D A. & MARSH, S. (1975). A very simple method for recording ganglion depolarization. *J. Physiol., Lond.*, **246**, 24–25P.

CURTIS, D.R., DUGGAN, A.W., FELIX, D. & JOHNSTON, G.A R (1971). Bicuculline an antagonist of GABA and synaptic inhibition in the spinal cord. *Brain Res.*, **32**, 69–96

CURTIS, D R., DUGGAN, A W., FELIX, D., JOHNSTON, G.A R. & McLENNAN, H. (1971). Antagonism between bicuculline and GABA in the cat brain. *Brain Res.*, **33**, 57–73.

CURTIS, D.R. & JOHNSTON, G A.R. (1974). Amino acid transmitters in the mammalian central nervous system. *Ergeb. der Physiol.*, **69**, 97–188.

CURTIS, D.R & WATKINS, J.C. (1960). The excitation and depression of spinal neurones by structurally related amino acids. *J. Neurochem.*, **6**, 117–141.

DE GROAT, W.C. (1970). The actions of γ-aminobutyric acid and related amino acids on mammalian autonomic ganglia *J. Pharmac. exp. Ther.*, **172**, 384–396.

DE GROAT, W C. (1972). GABA-depolarization of a sensory ganglion: antagonism by picrotoxin and bicuculline. *Brain Res.*, **38**, 429–432.

DE GROAT, W.C, LALLEY, P.M & SAUM, W.R. (1972). Depolarization of dorsal root ganglia in the cat by GABA and related amino acids: antagonism by picrotoxin and bicuculline. *Brain Res.*, **44**, 273–277.

DRAY, A (1975). Comparison of bicuculline methochloride with bicuculline and picrotoxin as antagonists of amino acid and monoamine depression on neurones in the rat brain stem. *Neuropharmacology*, **14**, 887–891.

GODFRAIND, J.M, KRNJEVIĆ, K., MARETIĆ, H., & PUMAIN, R. (1973). Inhibition of cortical neurones by imidazole and some derivatives. *Can. J. Physiol. Pharmac.*, **51**, 790–797.

HAAS, H.L., ANDERSON, E G & HOSLI, L (1973). Histamine and metabolites: their effects and interactions with convulsants on brain stem neurones. *Brain Res.*, **51**, 269–278.

HECHT, A.R. & HENEKA, H. (1949). A new high toxicity compound from sulphamide and formaldehyde. *Angew. Chem.*, **61**, 365–366.

HILL, R.G & SIMMONDS, M A (1973). A method for comparing the potencies of γ-aminobutyric acid antagonists on single cortical neurones using micro-iontophoretic techniques. *Br. J. Pharmac.*, **48**, 1–11.

IVERSEN, L L. & NEAL, M.J. (1968). The uptake of [^{3}H]-GABA by slices of rat cerebral cortex. *J. Neurochem.*, **15**, 1141–1149.

JOHNSTON, G.A.R., BEART, P.M., CURTIS, D R., GAME, C J A, McCULLOCH, R.M. & MacLACHLAN, R.M. (1972). Bicuculline methochloride as a GABA antagonist. *Nature, New Biol.*, **240**, 219–220.

JOHNSTON, G.A.R., CURTIS, D R., BEART, R M, GAME, C J.A., McCULLOCH, R.M. & TWITCHIN, B. (1975). *Cis* and *trans*-4-aminocrotonic acid as GABA analogues of restricted conformation. *J. Neurochem*, **24**, 157–160.

JONES, G.P. & PAULING, P.J. (1975). The crystal and molecular structure of *trans*-4-aminocrotonic acid. *J. Chem. Soc., Perkin Transactions II*, 1059–1062.

JONES, G.P. & PAULING, P J (1976a). The crystal and molecular structure of 4-aminotetrolic acid. *J. Chem. Soc., Perkin Transactions II*, (in press).

JONES, G.P. & PAULING, P.J. (1976b). The crystal and molecular structure of imidazole-4-acetic acid hydrochloride. *J. Chem. Soc., Perkin Transactions II*, (in press).

KIER, L.B, GEORGE, J.M. & HÖLTJE, H.D (1974). Structure-activity studies on γ-aminobutyric acid-like agents. *J. Pharm. Sc.*, **63**, No. 9, 1435–1439.

KLYNE, W & PRELOG, V. (1960). Description of steric relationships across single bonds. *Experientia*, **16**, 521–523.

KRNJEVIĆ, K. & PHILLIS, J.W. (1963). Actions of certain amines on cerebral cortical neurones. *Br. J. Pharmac. Chemother.*, **20**, 471–490.

MUSASHI, A. (1954). Synthese der γ-amino-crotonsaure und der γ-amino-buttersäure. *Hoppe Seylers Z. physiol. Chem.*, **297**, 71–73.

PHILLIS, J.W., TEBECIS, A.K. & YORK, D.H. (1968). Histamine and some antihistamines: their actions on cerebral cortical neurones. *Br. J. Pharmac.*, **33**, 426–440.

SANGIAH, S., BOROWITZ, J.L. & YIM, G K.W. (1974). Actions of GABA, picrotoxin, and bicuculline on adrenal medulla. *European J. Pharmac.*, **27**, 130–135.

TOMITA, K., HIGASHI, H. & FUJIWARA, T (1973). The crystal and molecular structure of γ-aminobutyric acid (GABA), a nervous inhibitory transmitter. *Bull. Chem. Soc. Japan*, **46**, 2199–2204.

WALSH, J.W, BOWERY, N G, BROWN, D A & CLARK, J (1974). Metabolism of γ-aminobutyric acid (GABA) by peripheral nervous tissue. *J. Neurochem.*, **22**, 1145–1147.

YOUNG, J.A.C., BROWN, D.A, KELLY, J S. & SCHON, F (1973) Autoradiographical localisation of sites of [^{3}H]-γ-aminobutyric acid accumulation in peripheral autonomic ganglia. *Brain Res.*, **63**, 479–486.

ZUKIN, S.R., YOUNG, A B. & SNYDER, S.H. (1974). Gamma-aminobutyric acid binding to receptor sites in the rat central nervous system. *Proc. Nat. Acad. Sci. U.S.A.*, **71**, 4802–4807.

(Received September 24, 1975.
Revised November 10, 1975.)

Br. J. Pharmac. (1976), **56**, 331–338

INVESTIGATION OF OCCURRENCE OF TOLERANCE TO BRONCHODILATOR DRUGS IN CHRONICALLY PRETREATED GUINEA-PIGS

A.A. ANDERSON & G.M. LEES

Department of Pharmacology, University Medical Buildings, Foresterhill,
Aberdeen AB9 2ZD

1 The actions of sympathomimetic amines on isolated preparations of atria, trachea and ileum were studied *in vitro* in guinea-pigs, which had been pretreated for 5 or 12 days, by subcutaneous injection, with adrenaline (5 μg/kg), salbutamol (0.5 μg/kg), methoxamine (250 μg/kg) or saline (0.9% w/v NaCl solution).

2 In the trachea, a decrease in sensitivity (tolerance) to the relaxant effect of adrenaline was induced by pretreatment, for 12 but not for 5 days, with adrenaline. In these animals, cross-tolerance to isoprenaline or salbutamol was not observed. Tolerance to the relaxant actions of adrenaline, isoprenaline or salbutamol was not observed after pretreatment with salbutamol.

3 In the trachea, pretreatment with methoxamine or adrenaline for 12 days did not change the sensitivity to the α-adrenoceptor-mediated contractor action of methoxamine.

4 In the atria from those guinea-pigs pretreated with adrenaline or salbutamol, there was no reduced sensitivity to the β-adrenoceptor agonist actions of adrenaline, isoprenaline or salbutamol. In animals pretreated with methoxamine or adrenaline, there was no observable tolerance or cross tolerance to methoxamine with respect to its α-adrenoceptor-mediated positive inotropic action in the atria and no unequivocal evidence of a reduced sensitivity to that action of adrenaline.

5 It was confirmed that the twitch-like contractions of the longitudinal muscle of the electrically stimulated ileum were inhibited by sympathomimetic amines acting on α- and β-adrenoceptors. There was no reduced sensitivity to the inhibitory actions of noradrenaline or isoprenaline on the twitch of ileum isolated from animals pretreated with adrenaline, salbutamol or methoxamine for 5 or 12 days.

6 From our results on these three preparations from the same animals, it is concluded that generalizations regarding changes in sensitivity to sympathomimetic amines following their prolonged administration should not be made in any one species.

Introduction

Between 1959 and 1966, mortality from bronchial asthma, particularly in younger age groups, increased three-fold in England and Wales (Speizer, Doll & Heaf, 1968). This has been attributed to excessive use of sympathomimetic bronchodilator agents administered by pressurized aerosol (Speizer, Doll, Heaf & Strang, 1968). In 72% of cases, the β-adrenoceptor stimulant used was isoprenaline.

Conolly, Davies, Dollery & George (1971) observed tolerance to the bronchodilator actions of isoprenaline *in vivo* in guinea-pigs. This was confirmed by Bouhuys, Douglas & Lewis (1972), but not by Pun, McCulloch & Rand (1971). Tolerance to the chronotropic actions of isoprenaline has also been reported as occurring in man and dogs (Conolly *et al.*, 1971) and in cats (Atkinson & Rand, 1968). More recently, however, Minatoya & Spilker (1975) did not observe tolerance to the chronotropic or bronchodilator actions of isoprenaline in *in vivo* experiments on healthy dogs. In a study of the ability of isoprenaline infusions to protect hypoxic dogs from the cardiotoxic effects of isoprenaline, McDevitt, Shanks & Swanton (1974) found no evidence of significant tolerance.

In a different approach, Benoy, El Fellah, Schneider & Wade (1975) observed that pretreatment of guinea-pigs three times daily with adrenaline given subcutaneously reduced the responsiveness to adrenaline of the isolated, perfused, histamine-constricted lungs of these animals. Moreover, they found that the longer the pretreatment period and the higher the dose of bronchodilator used in the pretreatment, the greater was the degree of tolerance developed.

The obvious controversy as to whether tolerance may develop in different tissues to β-adrenoceptor agonists might be resolved by studying quantitatively several actions of sympathomimetic bronchodilator drugs in tissues from the same animal after chronic pretreatment with one of these drugs. In view of the fact that salbutamol, unlike adrenaline, is much more active on bronchial smooth muscle than on cardiac muscle (Cullum, Farmer, Jack & Levy, 1969; Farmer, Levy & Marshall, 1970; Daly, Farmer & Levy, 1971), it was of interest to observe whether tolerance would also develop in animals pretreated with salbutamol. Furthermore, since α-adrenoceptors have been shown to mediate contractions of bronchial smooth muscle (Takagi, Osada, Takayanagi & Taga, 1967; Everitt & Cairncross, 1969; Persson & Johnson, 1970) and adrenaline is a powerful agonist on both types of adrenoceptor, the possibility of the development of tolerance to α-adrenoceptor-mediated contractions of trachea was also investigated.

Methods

Animal pretreatment

(a) Attempted induction of β-adrenoceptor tolerance. A total of 36 albino guinea-pigs, of either sex and of 310–580 g body weight, were pretreated subcutaneously, three times daily at (09 h 00 min, 14 h 00 min and 20 h 00 min) for either 5 or 12 days with 0.9% w/v NaCl solution (saline), adrenaline (5 µg/kg) or salbutamol (0.5 µg/kg).
(b) Attempted induction of α-adrenoceptor tolerance. A total of 12 male guinea-pigs of 310–530 g body weight were pretreated subcutaneously, three times daily for 12 days with saline, adrenaline (5 µg/kg) or methoxamine (250 µg/kg).

In both pretreatment regimens, these doses were chosen on the basis of results obtained in experiments which showed that salbutamol was about ten times more potent and methoxamine about fifty times less potent than adrenaline in causing, respectively, relaxations and contractions of spiral preparations of trachea (Caswell, Cormack & Lees, unpublished observations). Dilutions of drugs were made up freshly with sterile saline before each pretreatment. The volume injected was 0.15–0.3 ml, depending on the body weight. Each animal showed a satisfactory and steady gain in weight throughout the pretreatment period. They were fed on a diet of Coney Brand pellets supplemented with greens and carrots three times a week; water, containing ascorbic acid (1 g/l), was available *ad libitum*.

Solutions and drugs

Isolated preparations were in contact with a physiological salt solution of the following composition (mM): NaCl 118.2, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 25.0, glucose 11.2. The bath temperature was maintained at 37°C ± 0.5°C and the solution gassed with a mixture of 95% O_2 and 5% CO_2.

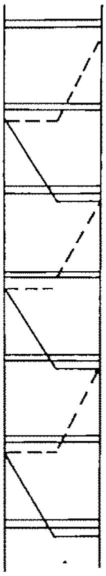


Figure 1 Diagrammatic representation of trachea showing position of cuts through muscle and cartilaginous rings. Solid lines indicate cuts on posterior surface of trachea, dashed lines show cuts on anterior aspect.

The following drugs were used: acetylcholine chloride (BDH); (—)-adrenaline (base) (Koch Light); isoprenaline hydrochloride (Winthrop); (±)-methoxamine hydrochloride (Burroughs Wellcome); (—)-noradrenaline acid tartrate (Koch Light); salbutamol (base) (Allan & Hanburys); (±)-propranolol hydrochloride (ICI); phentolamine hydrochloride or mesylate (Ciba).

Stock solutions of adrenaline were made up in 0.01 M HCl; acetylcholine stock solutions were prepared in 5% NaH_2PO_4. Other stock solutions were made up in a modified Krebs solution of the following composition (mM): NaCl 143, KCl 4.75, $CaCl_2$ 2.54. All were stored at −23°C. Dilutions of acetylcholine were acidified with HCl to bring the pH to 4. All other dilutions of stock solutions were made in the modified Krebs solution to which 0.2 mg/ml ascorbic acid was added to prevent oxidation. Final bath concentrations of all drugs are expressed in terms of the free base.

Preparations

The guinea-pigs were stunned by a blow on the head and bled from the left femoral artery. The heart was removed as quickly as possible and placed in warm Krebs solution. The atria were then carefully dissected from the ventricles and mounted in Krebs solution in an organ bath. A tension of 0.5 g was applied, isometric contractions of the isolated atria being detected by strain gauge transducers (Ether, type UFI ± 2 ozs) and displayed on a Grass Polygraph (Model 7).

The trachea was carefully excised and cut spirally in the manner indicated in Figure 1: this method of cutting the trachea in steeper spirals results in an increased sensitivity. The tracheal spiral was mounted in Krebs solution at 37°C in an organ bath and a tension of 1.0 g applied. Isometric contractions and relaxations were recorded by means of strain gauge transducers connected to a Servoscribe (R.E. 250) potentiometric pen recorder. The wet weight of the tracheal strips, which was measured on completion of the experiment, was found to be 67.3 ± 1.3 mg (mean with s.e. mean; $n=49$).

The terminal portion of the ileum was used after the 20 cm nearest to the ileo-caecal junction had been discarded because of the presence of excitatory α-adrenoceptors near the ileo-caecal junction (Munro, 1953). The depressant actions of noradrenaline, isoprenaline, salbutamol and methoxamine were tested on 5–6 cm segments stimulated electrically with coaxial electrodes, essentially according to the method of Paton (1955). Isometric, twitch-like contractions of the ileum were recorded by means of a strain gauge transducer and displayed on a potentiometric pen recorder. The stimuli were 1.2–1.3 times maximal rectangular pulses of 0.4–0.5 ms duration at a frequency of 0.1 Hz; checks were made throughout an experiment to ensure that the stimuli were supramaximal.

In all experiments, the preparations were allowed to recover for 1–1.5 h before starting the experiment. In the atria, dose cycles of not less than 20 min were employed in order to achieve consistent responses to adrenaline and isoprenaline and of not less than 30 min for salbutamol and methoxamine. In the trachea, dose cycles used for adrenaline and methoxamine were 25 min and 45 min respectively. All other agonists had dose cycles in this range. These times were based on the results of previous experiments.

Expression of results

Trachea: Since the resting tension was not constant but sometimes varied markedly, an allowance was made for these changes by measuring the change in muscle tension induced by a drug as a percentage of the tension existing immediately before exposure to the drug, i.e. these results are expressed as percentage loss or gain in tension.

Atria: Changes in rate and force of contraction were always expressed as percentage increases. After responses to salbutamol, however, the rate and contractile force never returned to the values observed before exposure to salbutamol. Thus, apparently smaller increases in heart rate and force of contraction were obtained because of the elevated 'background' values; an apparent bell-shaped dose-response relationship was frequently observed. The problem was overcome by assuming values of rate and contractile force prior to the first injection of salbutamol to be control values for the first and subsequent injections of salbutamol.

Ileum: The depressant action of drugs on the electrically-induced contraction of the longitudinal muscle was always expressed as percentage reduction of the twitch height before exposure to the drug (% inhibition of the twitch).

Statistical analysis

Decreases in responsiveness (tolerance) to adrenaline, salbutamol or methoxamine would appear as a rightward shift of the log dose-response curve compared with saline pretreated animals. In every case, the dose-response curves were analysed by Student's t test in order to determine whether a statistically significant displacement had occurred. Differences were taken to be significant when $P<0.05$, where P indicates the probability of two points being similar.

Results

β-Adrenoceptor-mediated responses in trachea

Adrenaline (10–300 nM), isoprenaline (3–300 nM) and salbutamol (1–300 nM) were found to induce relaxation of the preparations. There was no significant difference between the degree of relaxation induced by adrenaline in animals pretreated for 5 days with saline or adrenaline (Figure 2a). Pretreatment with adrenaline for 12 days, however, caused a significant displacement of the adrenaline dose-response curve to the right for the corresponding curve for trachea from saline-pretreated animals (Figure 2b). No statistically significant difference was observed between the relaxations induced by isoprenaline or salbutamol in adrenaline-pretreated and control animals ($P>0.1$) i.e. no significant cross-tolerance to these drugs was observed.

Responses to salbutamol in the trachea were unchanged after 5 and 12 days pretreatment with salbutamol as compared with saline-pretreated controls (Table 1 a & b). Cross-tolerance to adrenaline or isoprenaline was not observed.

β-Adrenoceptor-mediated responses in atria

Concentrations of adrenaline, isoprenaline and salbutamol were used which induced 10–60% increases in rate of beating; these concentrations

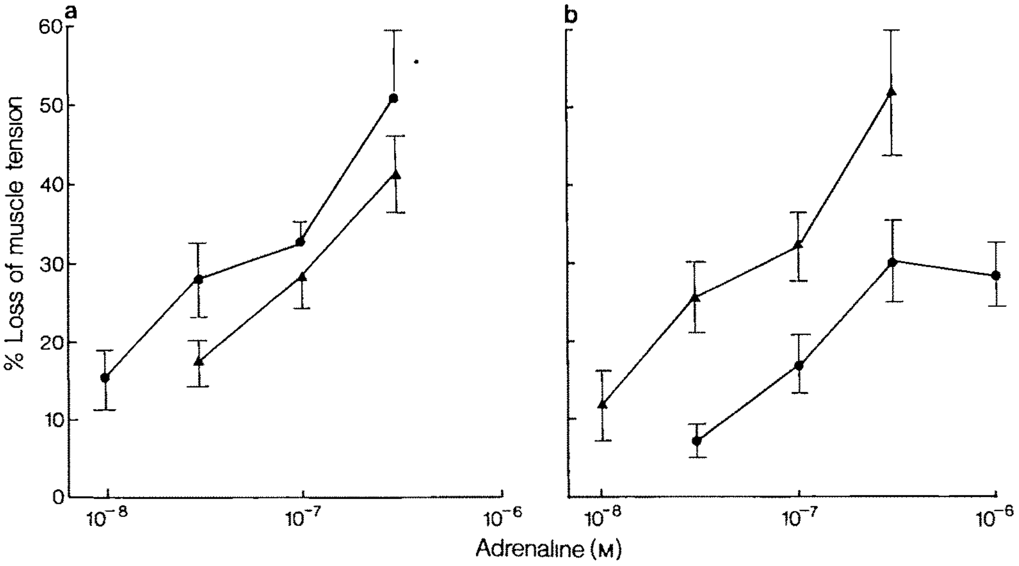


Figure 2(a) Relaxation induced by adrenaline in tracheal smooth muscle from guinea-pigs pretreated for 5 days with saline (▲) or adrenaline (●) Each point represents the mean and the vertical bars the s.e. mean of 6 observations for 6 animals; there were only 2 observations of responses to 300 nM adrenaline in adrenaline-pretreated animals. (b) Relaxation induced by adrenaline in tracheal smooth muscle from guinea-pigs pretreated for 12 days with saline (▲) or adrenaline (●) Points and vertical bars represent means with s.e. mean of 4–7 observations; there were only 2 observations of responses to 10 nM adrenaline in saline-pretreated animals. In (a) and (b) ordinates: loss of muscle tension (% control) (see Methods section: Expression of results); abscissae. concentration of adrenaline (M).

Table 1(a) Relaxation of tracheal smooth muscle by salbutamol after 5 day pretreatment with saline or salbutamol (0.5 μg/kg s.c.)

	% Loss of bronchial muscle tension		*Statistical analysis*	
Salbutamol (nM)	*Saline-pretreatment*	*Salbutamol-pretreatment*	t	P
1	11.3±1.3(6)	—	—	—
3	16.2±1.5(6)	14 1±2.4(2)	0.74	0 49
10	25.8±3 2(6)	27.9±3.0(4)	−0.48	0 64
30	34.9±10.1(2)	53.8±2.7(6)	−1.81	0 10

Values are of mean with s e mean. The numbers in parentheses refer to the number of observations made in each case.

Table 1(b) Relaxation of tracheal smooth muscle by salbutamol after 12 day pretreatment with saline or salbutamol (0 5 μg/kg s.c.).

	% Loss of bronchial muscle tension		*Statistical analysis*	
Salbutamol (nM)	*Saline-pretreatment*	*Salbutamol-pretreatment*	t	P
1	13.2±2 0(2)	—	—	—
3	22.2±5.5(4)	12.8±4.3(5)	1.35	0 23
10	29.5±8.0(4)	19.3±4 4(7)	1 12	0.29
30	33.6±7.7(4)	30.7±5 2(8)	0.31	0.76
100	—	31 9±7.6(6)	—	—

Values are of mean with s.e. mean. The numbers in parentheses refer to the number of observations made in each case induced 25–400% increases in the force of isometric contractions of the atria. The relative potencies of adrenaline, isoprenaline and salbutamol were similar with respect to their ability to induce positive chronotropic and inotropic responses in the guinea-pig atria: isoprenaline was about 35 times more potent than adrenaline and about 450 times more potent than salbutamol.

In guinea-pigs pretreated with adrenaline for 12 days the chronotropic responses to adrenaline were not different from those of animals pretreated with saline for the same period ($P > 0.3$); similarly, the inotropic responses were unchanged ($P > 0.12$). In animals pretreated for 5 and 12 days with salbutamol, the chronotropic and inotropic responses to salbutamol were unchanged from those observed in atria from saline-pretreated controls ($P > 0.21$). As in the trachea, the phenomenon of cross-tolerance was not observed.

α-Adrenoceptor-mediated responses in trachea

Methoxamine has a direct α-adrenoceptor agonist action (Trendelenburg, 1972) and β-adrenoceptor blocking activity (Patil, Tye & La Pidus, 1967). In the concentration range 3–1000 nM, it induced slow, concentration-dependent contractions of the isolated tracheal strip, which were antagonized by phentolamine (300 nM). Acetylcholine (0.1–10 μM) also induced contractions of the trachea but these were much faster and were followed by a slower relaxation to less than basal tension; it required about 10 min for the smooth muscle tension to return to normal after these relaxations, which were not further investigated, attention being focussed on the contractions. Phentolamine (300 nM) was observed to potentiate the acetylcholine-induced contraction by $58.0 \pm 11.0\%$ (mean with s.e. mean, $n = 10$).

Contractor responses to methoxamine in four animals pretreated for 12 days with methoxamine or adrenaline were not significantly different from responses in saline-pretreated animals ($P > 0.05$). Pretreatment of animals for 12 days with either adrenaline or methoxamine did not modify the action of acetylcholine on the tracheal strip ($P > 0.12$).

α-Adrenoceptor-mediated responses in atria

Although there is no evidence that changes in rate and force of beating of the human heart are mediated by α-adrenoceptors, we found in guinea-pig isolated atria that methoxamine (1–300 μM) had a positive inotropic action but no positive chronotropic effect. This inotropic response was unaffected by propranolol (0.75 μM) but was abolished by phentolamine (300 nM), observations which are in agreement with the findings of Govier (1968). The chronotropic action of adrenaline (0.1–10 μM) was abolished and its inotropic action was greatly reduced by propranolol (0.75 μM): the remaining inotropic effect of adrenaline was abolished by phentolamine (300 nM). In saline-pretreated animals, methoxamine was about one thousand times less effective than adrenaline in inducing a positive inotropic action. To ensure that the inotropic responses to adrenaline and methoxamine were mediated purely by α-adrenoceptors and not partly by β-adrenoceptors, the responses were measured in the presence of a concentration of propranolol that was sufficient to block the chronotropic and inotropic actions of isoprenaline (100 nM), isoprenaline having a powerful action on β-adrenoceptors but almost no action on α-adrenoceptors. It was found that positive inotropic responses mediated by α-adrenoceptors were much smaller than those mediated by β-adrenoceptors. In the four guinea-pigs pretreated for 12 days with methoxamine or adrenaline there was, however, no unequivocal evidence that a lowered sensitivity had been induced.

α- and β-Adrenoceptor-mediated responses in ileum

The inhibitory effects of catecholamines on coaxially-stimulated guinea-pig isolated ileum are well documented and analysed (Kosterlitz, Lydon & Watt, 1970); both α- and β-adrenoceptors are involved in the depression of the twitch. This preparation was, therefore, chosen as a suitable one for examination of possible changes in responsiveness to sympathomimetic amines in the chronically pretreated guinea-pigs.

In confirmation of the findings of Kosterlitz *et al.* (1970), the minimum effective concentration of noradrenaline that produced a reduction of the twitch was 10 nM. Isoprenaline and noradrenaline, rather than adrenaline, were chosen as standard agonists because, in this preparation, noradrenaline has a pronounced β-adrenoceptor agonist action, whereas adrenaline acts predominantly on α-adrenoceptors (Kosterlitz *et al.*, 1970; Lees, unpublished observations). It had been assumed previously that isoprenaline produces an inhibition of the twitch due to an action on β-adrenoceptors. However, we found in some ileum preparations that, in the presence of propranolol (0.1–5 μM) there remained a small inhibitory effect which was reversed or prevented by phentolamine (0.1–2.5 μM); in some animals, therefore, up to about 25% of the inhibitory effect of isoprenaline is mediated by α-adrenoceptors. In the presence of isoprenaline or noradrenaline, in concentrations which produced maximal and half-maximal inhibition of the electrically-induced contractions, it was found that acetylcholine-induced contractions of the longitudinal muscle were depressed by these catecholamines and that this depression was partially reversed by phentolamine (0.1–0.5 μM) given

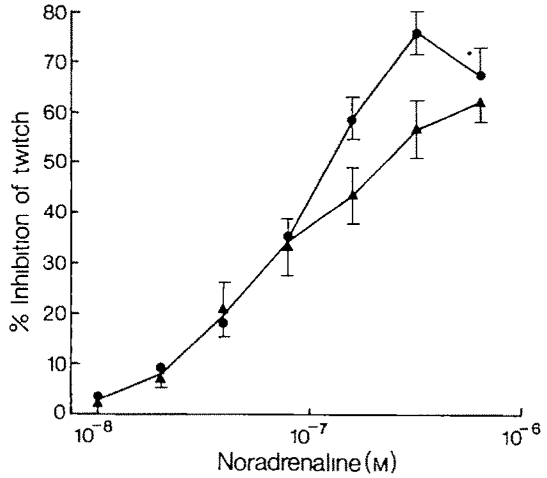


Figure 3 Depressant effect of noradrenaline on the responses to electrical stimulation (0.1 Hz) of the ileum from guinea-pigs pretreated for 12 days with saline (▲) or adrenaline (●). Ordinate scale: inhibition of contraction of longitudinal muscle (% control). Abscissa scale concentration of noradrenaline (M). Points and vertical bars indicate means with s.e. mean of 7–12 observations; there were 3–4 observations of responses to 640 nM noradrenaline

alone or during exposure to (—)-propranolol (1 μM). These results provide additional evidence that isoprenaline may act on α-adrenoceptors and that these receptors are probably present on the smooth muscle, as in the rabbit ileum (Bowman & Hall, 1970), in addition to being on neuronal elements (Kosterlitz *et al.*, 1970).

In animals pretreated with adrenaline for 5 and 12 days, the twitch of the longitudinal muscle was as greatly and as readily inhibited by noradrenaline as in the appropriate controls, with the exception of the response to the highest concentrations of noradrenaline (160 and 320 nM) which produced a significantly ($P < 0.05$) greater inhibition of the twitch of ilea from guinea-pigs pretreated with adrenaline for 12 days (Figure 3). This unexpected result is probably explained by the additional finding that the sensitivity to near maximally-effective concentrations of isoprenaline was significantly increased in these animals: the inhibition produced by 160 nM isoprenaline in saline controls was 14.3 ± 2.9% ($n = 8$), whereas in 12 day adrenaline-pretreated animals it was 24.1 ± 3.3% ($n = 11$) ($P < 0.05$). There were no significant changes in the sensitivity of the ileum to noradrenaline or isoprenaline in salbutamol- or methoxamine-pretreated guinea-pigs. Interestingly, salbutamol (0.64–5.12 μM) itself produced an inhibition of the longitudinal muscle contraction but it was at least 30 times less potent than isoprenaline. Methoxamine could not be successfully used as a standard α-adrenoceptor agonist in this preparation owing to the long-lasting dose-dependent contraction of the longitudinal muscle it induced; excitatory responses to noradrenaline were rarely seen and were very small.

Discussion

Over the last few years, studies of tolerance (i.e. a reduced responsiveness) to sympathomimetic bronchodilator drugs have been approached in two major ways, over short periods of only a few hours and over much longer periods, in clinical studies. However, in both of these approaches reports of whether tolerance develops or not in either man or experimental animals have been widely contradictory. More recently, Benoy *et al.* (1975) reported that tolerance to the bronchodilator action of adrenaline developed following pretreatment of guinea-pigs with adrenaline (5 μg/kg) for only three days. In an attempt to confirm and extend the findings of these authors, the same conditions of pretreatment were observed. The concentration of adrenaline which was used for pretreatment is approximately the same dose (on a weight per kilogram basis) as a patient with bronchial asthma might receive in an acute attack, so this must be regarded as a large dose when given regularly.

In the present experiments, salbutamol was found to be equipotent with isoprenaline in inducing relaxations of the tracheal muscle but 450 times less potent than isoprenaline in its chronotropic and inotropic actions on the isolated atria. Similarly, adrenaline was 10 times less potent than salbutamol in its action on the trachea but 13 times more potent on the atria. The results also indicate that tolerance to adrenaline in its action on bronchial smooth muscle developed after 12 days pretreatment but not after 5 days; in contrast, Benoy *et al.* (1975) observed tolerance after a much shorter pretreatment period and after 14 days pretreatment, the adrenaline dose-response curve was almost horizontal. The discrepency could be due to differences in experimental conditions; for example, Benoy *et al.* (1975) measured changes in the flow rate of saline solution in an isolated perfused lung preparation after administration of a bronchodilator drug, whereas, in the present study, relaxations of tracheal smooth muscle where measured. It is possible that drugs such as adrenaline, isoprenaline and theophylline have a more pronounced action on the muscle of the lower respiratory tract than of the upper respiratory tract. It is interesting that, although Benoy *et al.* (1975) reported cross-tolerance to adrenaline, isoprenaline and theophylline with respect to their bronchodilator actions, no evidence of cross-tolerance to isoprenaline or salbutamol was obtained in our experiments. We did not observe tolerance to the action of salbutamol on tracheal smooth muscle even after 12 days pretreatment with salbutamol. This would suggest that, if a reduced sensitivity to the bronchodilator action of salbutamol can be induced, it develops more slowly than it does to adrenaline. In this connection, the results of a study by Sims (1974) in patients with reversible airways disease are of interest. These patients received either salbutamol orally for four weeks or salbutamol by aerosol inhaler for two weeks: the improvement in Forced Expiratory Volume in 1 s (FEV_1) induced by salbutamol in patients after these treatments was not significantly different from the improvement in FEV_1 recorded before treatment. In similar clinical studies, Gibson & Tattersfield (1972) and Parker, Choo-Kang, Cooper, Cameron & Grant (1971) did not observe any decrease in sensitivity following treatment with salbutamol, given orally, for four weeks.

Since Castro de la Mata, Penna & Aviado (1962) demonstrated the existence of α-adrenoceptors which were considered responsible for bronchoconstriction in the anaesthetized dog, much attention has been focussed on the existence of excitatory α-adrenoceptors in the respiratory tract. Evidence has been presented for the presence of these receptors in human bronchial musculature (Prime, Bianco, Griffin & Kamburoff, 1972; Gaddie, Legge, Petrie & Palmer, 1972). We were unable to demonstrate a decreased sensitivity to the α-adrenoceptor-mediated contractor response to methoxamine in tracheal smooth muscle in guinea-pigs pretreated for 12 days with adrenaline or methoxamine.

If it is assumed that responses mediated by the α-adrenoceptor, in addition to the β-adrenoceptor, play an important role in bronchial asthma, asthmatic patients may become tolerant to the bronchodilator action of adrenaline but not to the weaker bronchoconstrictor action. In this situation, the asthmatic condition would naturally be aggravated. This may be the basis for the improvement in airway conductance in asthmatic patients who have been given α-adrenoceptor blocking agents (Griffin, Kamburoff, Prime & Abbab, 1972).

Tolerance to the β-adrenoceptor actions of adrenaline or salbutamol on the isolated atria was not observed. This is in agreement with the results of clinical studies by Pierson & Grieco (1969), Cookson & Reid (1963) and Kingsley, Littlejohns & Prichard (1972) and of animal studies by Minatoya & Spilker (1975).

From the results obtained in the coaxially-stimulated ileum preparations, it is concluded that pretreatment of the guinea-pigs for 12 days with adrenaline or salbutamol did not lead to a diminished sensitivity of the ileum to β-adrenoceptor-mediated responses; indeed, there was evidence to suggest that the sensitivity was enhanced in the adrenaline-pretreated group, the tracheal muscle of these same animals showing a reduced responsiveness to the β-adrenoceptor-mediated relaxant action of adrenaline. In agreement with the findings for the trachea, there was no evidence of a changed sensitivity in the ileum to the α-adrenoceptor-mediated actions of noradrenaline in animals pretreated with adrenaline, methoxamine or salbutamol.

On the basis of our results obtained in three preparations each from the same guinea-pig, it is clear that generalizations regarding quantitative effects of sympathomimetic amines acting via β-adrenoceptors should not be made for any one species.

We should like to thank Allan & Hanburys for a gift of salbutamol and ICI for propranolol.

References

ATKINSON, J.M. & RAND, M.J. (1968). Mutual suppression of cardiovascular effects of some β-adrenoceptor agonists in the cat. *J. Pharm. Pharmac.*, **20**, 916–922.

BENOY, C.J, EL FELLAH, M S, SCHNEIDER, R. & WADE, O L (1975). Tolerance to sympathomimetic bronchodilators in the guinea-pig lungs. *Br. J. Pharmac.*, **53**, 463P.

BOUHUYS, A., DOUGLAS, J.S. & LEWIS, A.J (1972). Hypersensitivity to adrenoceptor agents in the guinea-pig in vitro and in vivo. *Br. J. Pharmac.*, **46**, 520–521P.

BOWMAN, W.C & HALL, M.T. (1970). Inhibition of rabbit intestine mediated by α- and β-adrenoceptors. *Br. J. Pharmac.*, **38**, 399–415.

CASTRO DE LA MATA, R., PENNA, M. & AVIADO, D.M. (1962). Reversal of sympathomimetic bronchodilation by Dichloro-isoproterenol. *J. Pharmac. exp. Ther.*, **135**, 197–203.

CONOLLY, M.E., DAVIES, D.S, DOLLERY, C.T. & GEORGE, C.F. (1971). Resistance to β-adrenoceptor stimulants (a possible explanation for the rise in asthma deaths). *Br. J. Pharmac.*, **43**, 389–402.

COOKSON, D U. & REID, C.E. (1963). A comparison of the effects of isoproterenol in the normal and asthmatic subject. *Am. Rev resp. Dis.*, **88**, 636–643.

CULLUM, V.A, FARMER, J.B, JACK, D. & LEVY, G.P. (1969). Salbutamol: a new, selective β-adrenoceptive receptor stimulant. *Br. J. Pharmac.*, **35**, 141–151.

DALY, M.J., FARMER, J.B. & LEVY, G.P. (1971). Comparison of the bronchodilator and cardiovascular actions of salbutamol, isoprenaline and orciprenaline in guinea-pigs and dogs. *Br. J. Pharmac.*, **43**, 624–638.

EVERITT, B.J. & CAIRNCROSS, K.D. (1969). Adrenergic receptors in guinea-pig trachea. *J. Pharm. Pharmac.*, **45**, 451–462.

FARMER, J.B., LEVY, G.P. & MARSHALL, R.J. (1970). A comparison of the β-adrenoceptor stimulant properties of salbutamol, orciprenaline and soterenol with those of isoprenaline. *J. Pharm. Pharmac.*, **22**, 945–946.

GADDIE, J., LEGGE, J.S., PETRIE, G & PALMER, K.N.V. (1972). Effect of an α-adrenergic receptor blocking drug on histamine sensitivity in bronchial asthma. *Br. J. Dis. Chest.*, **66**, 141.

GIBSON, J. & TATTERSFIELD, A E (1972). The effects of oral salbutamol on response to inhaled isoprenaline in asthmatic subjects. *Bull. Physio.-path. resp.*, **8**, 657–658.

GOVIER, W.C (1968). Myocardial α-receptors and their role in the production of a positive inotropic effect by sympathomimetic agents. *J. Pharmac. exp. Ther.*, **159**, 82–90.

GRIFFIN, J P, KAMBUROFF, P.L, PRIME, F.J. & ABBAB, A.G. (1972). Thymoxamine and airways obstruction. *Lancet*, **1**, 1288.

KINGSLEY, P.J., LITTLEJOHNS, D.W. & PRICHARD, B.N.C. (1972). Isoprenaline-induced tachycardia in man. *Br. J. Pharmac.*, **46**, 539–540P.

KOSTERLITZ, H.W., LYDON, R.J & WATT, A.J. (1970). The effects of adrenaline, noradrenaline and isoprenaline on inhibitory α- and β-adrenoceptors in the longitudinal muscle of the guinea-pig ileum. *Br. J. Pharmac.*, **39**, 398–413.

McDEVITT, D.G., SHANKS, R.G. & SWANTON, J.G. (1974). Further observations on the cardiotoxicity of isoprenaline during hypoxia. *Br. J. Pharmac.*, **50**, 335–344.

MINATOYA, H. & SPILKER, B.A. (1975). Lack of cardiac or bronchodilator tachyphylaxis to isoprenaline in the dog. *Br. J. Pharmac.*, **53**, 333–340.

MUNRO, A.F. (1953). Effect of autonomic drugs on the responses of isolated preparations from the guinea-pig intestine to electrical stimulation. *J. Physiol., Lond.*, **120**, 41–52.

PARKER, S S., CHOO-KANG, Y.F.J., COOPER, E.J., CAMERON, S J. & GRANT, I.W.B. (1971). Bronchodilator effect of oral salbutamol in asthmatics treated with corticosteroids. *Br. med. J.*, **4**, 139–142.

PATIL, P N., TYE, A. & LA PIDUS, J.B. (1967). Steric aspects of adrenergic drugs. V. Beta-adrenergic blocking effects of optical isomers of methoxamine and isopropylmethoxamine. *J. Pharmac. exp. Ther.*, **156**, 445–451.

PATON, W.D.M. (1955). The response of the guinea-pig ileum to electrical stimulation by coaxial electrodes. *J. Physiol., Lond.*, **127**, 40–41P.

PERSSON, J & JOHNSON, B. (1970). Adrenergic receptors in the guinea-pig trachea and lung. *Acta pharmac. tox.*, **28**, 49–56.

PIERSON, R.N Jr. & GRIECO, M.H. (1969). Isoproterenol aerosol in normal and asthmatic subjects. Time relationship of pulmonary and haemodynamic responses. *Am. Rev. resp. Dis.*, **100**, 533–541.

PRIME, F.J., BIANCO, S., GRIFFIN, J.P & KAMBUROFF, P.L. (1972). The effects on airways conductance of α-adrenergic stimulation and blocking. *Bull. Physio-path. resp.*, **8**, 99.

PUN, L.-Q., McCULLOCH, M.W. & RAND, M J (1971). Bronchodilator effects of sympathomimetic amines given singly and in combination. *Eur. J. Pharmac.*, **14**, 140–149.

SIMS, B.A (1974). Investigation of salbutamol tolerance. *Br. J. Clin. Pharmac.*, **1**, 291–294.

SPEIZER, F.E., DOLL, R. & HEAF, P. (1968). Observations on recent increase in mortality from asthma. *Br. med. J.*, **1**, 335–339.

SPEIZER, F.E., DOLL, R., HEAF, P. & STRANG, L.B. (1968). Investigation into use of drugs preceding death from asthma. *Br. med. J.*, **1**, 339–343.

TAKAGI, K, OSADA, E., TAKAYANAGI, I & TAGA, F. (1967). Adrenergic receptors in some organs. *Arch. int. Pharmcodyn. Ther.*, **168**, 212–219.

TRENDELENBURG, U. (1972). Classification of sympathomimetic agents. In *Handbook of Experimental Pharmacology*, **33**, *Catecholamines*. ed. Blaschko, H. & Muscholl, E. pp. 336–362. Berlin: Springer-Verlag.

(Received November 12, 1975.)

PROCEEDINGS OF THE
British Pharmacological Society

17th-19th December, 1975

ROYAL COLLEGE OF SURGEONS

COMMUNICATIONS

In communications with more than one author, an asterisk (*) denotes the one who presented the work.

Selectivity of action of some C15-modified prostaglandins D

R.L: JONES

Department of Pharmacology, University of Edinburgh, 1 George Square, Edinburgh EH8 9JZ

Prostaglandin D_2 (PGD_2) raises the blood pressure of the anaesthetized sheep by direct vasoconstriction and is some 70 times more active than $PGF_{2\alpha}$ (Horton & Jones, 1974; Jones, 1975). From preliminary studies on prostaglandins of the F series, it appeared that certain C15-modified analogues of PGD_2 might exhibit a greater selectivity of action than PGD_2. The 15-methyl ether, 15(R), 15-oxo and 13,14-dihydro-15-oxo analogues of PGD_2 have therefore been prepared. Equipotent molar ratios ($PGD_2 = 1.0$) for these four compounds and the corresponding $PGF_{2\alpha}$ analogues as pressor agents in the sheep are shown in Table 1. It can be seen that the modified prostaglandins D exhibit high pressor activity whereas the corresponding PGE_2 analogues are much less active than PGE_2 as depressor agents.

In the rabbit PGD_2 and PGE_2 are potent pressor and depressor agents respectively and similar equipotent molar ratios for many of the compounds in Table 1 have been found. In the rat PGE_2 (100 ng/kg intra-aortically) lowers the blood pressure. PGD_2 elicits a small pressor response at doses 50 times greater (on a body weight basis) than those effective in the sheep. At higher doses (5 μg/kg), PGD_2 gives a biphasic response. However, PGD_2 15-methyl ether, 13,14-dihydro-15-oxo PGD_2 and 15-oxo $PGF_{2\alpha}$ produce only pressor responses. It is suggested that these C15-modified prostaglandins are devoid of the significant PGE-like depressor activity shown by PGD_2.

On the rabbit oviduct *in vivo*, $PGF_{2\alpha}$ raises the intra-luminal pressure (Horton & Main 1965); PGD_2 15-methyl ether was found to be 140 times

Table 1 Comparison of C15-modified prostaglandins on the sheep blood pressure (intra-aortic injection)

	Equipotent molar ratios		
	Pressor response		*Depressor response*
ω-side chain characteristics	*PGD_2 analogues*	*$PGF_{2\alpha}$ analogues*	*PGE_2 analogue*
15(S)-hydroxyl	1.0	69	1.0
15(S)-methyl ether	5.6	107	> 100
15(R)-hydroxyl	1.9*	69*	550
15-oxo	8.4†	13	> 300
13,14-dihydro-15-oxo	5.7	550	> 300

Each ratio is the mean of at least three determinations.
* Racemic mixture
† Major component is the 12,13-ene isomer.

less active than $PGF_{2\alpha}$. (±)15(R) PGD_2 and 13,14-dihydro-15-oxo PGD_2 produced no effect at doses 100 and 330 times respectively, the threshold dose of $PGF_{2\alpha}$. Finally on the isolated rabbit jejunum, where PGD_2 is about 5 times less active than $PGF_{2\alpha}$, PGD_2 15-methyl ether and (±)15(R) PGD_2 are at least 100 times and the two 15-oxo PGD analogues at least 500 times less active than $PGF_{2\alpha}$

Thus it would appear that simple chemical changes can be made at C15 which result in retention of PGD-like activity but loss of PGE- and PGF_{α}-like activities.

Prostaglandins were kindly supplied by I.C.I. Ltd and the Upjohn Co., Kalamazoo

References

HORTON, E.W & JONES, R.L. (1974). Biological activity of prostaglandin D_2 on smooth muscle. *Br J. Pharmac*, 52, 110-111P.

HORTON, E.W. & MAIN, I.H M. (1965) A comparison of the actions of prostaglandins $F_{2\alpha}$ and E_1 on smooth muscle. *Br J. Pharmac.*, **24**, 470-476

JONES, R.L. (1975). Actions of prostaglandins on the arterial system of the sheep. some structure–activity relationships *Br. J. Pharmac.*, **53**, 464P

Prostaglandins and changes in the gastric mucosal barrier and blood flow during indomethacin- and bile salt-induced mucosal damage

B.J.R. WHITTLE

Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons of England, London WC2A 3PN

The formation of gastric mucosal erosions by non-steroid anti-inflammatory drugs in the rat is greatly increased by the presence of bile acids in the gastric lumen (Semple & Russell, 1975), although this damage is prevented by prostaglandin methyl analogues (Whittle, 1975). Changes in gastric mucosal blood flow and in the resistance of the mucosa to acid back-diffusion (the mucosal 'barrier') have now been investigated as possible mechanisms underlying such erosion formation.

The gastric lumen of the urethane-anaesthetized rat was perfused with acidic saline (0.1-0.2 ml min^{-1}) and the loss of acid across the mucosa determined by titration. The potential difference (PD) across the mucosa, which is related to hydrogen- and sodium-ion flux and gives an indication of the integrity of the mucosal barrier (Chvasta & Cooke, 1972) was measured via calomel electrodes. Mucosal blood flow (MBF) was determined by [^{14}C]-aniline clearance (Main & Whittle, 1973).

During acid perfusion (100 mM HCl, pH 1), administration of sodium taurocholate (1 mg/ml, 2 mM) increased the acid-loss (from 0.48 ± 0.19 to 2.2 ± 0.3 μEq min^{-1} after 1 h; mean ± s.e mean, $n = 4$), lowered PD (by −10.4 ± 1.5 mV, $n = 8$) and increased MBF (to 340 ± 15% of basal, $n = 4$). The rise in MBF appeared to correlate with acid back-diffusion and may represent a protective mechanism of the mucosa, since few erosions were seen after the 3 h perfusion. Indomethacin (20 mg/kg i.v.), injected during acid perfusion, decreased MBF (by 30 ± 4% of basal, $n = 3$) but had no consistent effect on acid-loss or PD, and a low erosion 'score' was observed after 3 hours. However, during taurocholate perfusion, indomethacin reduced the elevated MBF (to 244 ± 17% of basal, $n = 4$) and led to a high incidence of erosions. The total acid-loss during taurocholate perfusion was 360 ± 50 μEq 3 h^{-1} ($n = 5$), and was 460 ± 40 μEq 3 h^{-1} ($n = 4$) following simultaneous administration of indomethacin (20 mg/kg s.c.)

The acid back-diffusion during combined taurocholate and indomethacin administration was reduced (20 ± 5%, $n = 8$) by the (15S)-15 methyl analogue of prostaglandin E_2 (5 $\mu g\ kg^{-1} h^{-1}$ s.c.), in a dose causing 53% inhibition of the erosions (Whittle, 1975). Intravenous infusion of the prostaglandin analogue (5 $\mu g\ kg^{-1}$ over 1 h) increased PD (by -6.7 ± 0.9 mV, $n = 3$) and MBF (by 66 ± 13% of basal, $n = 3$) following taurocholate or indomethacin administration, and during resting conditions. Exogenous prostaglandins may therefore prevent erosions by actions on both MBF and mucosal permeability However, the failure of parenteral indomethacin, alone, to markedly alter resting PD or acid-loss, in doses reducing mucosal prostaglandin levels (Main & Whittle,1975) may argue against a local role for endogenous prostaglandins in the maintenance of the rat mucosal barrier.

These results in the rat suggest that although a reduction in MBF, as observed with parenteral indomethacin, or an increased acid back-diffusion as seen with taurocholate, can lead to a low incidence of mucosal erosions, a combination of both produces extensive mucosal damage. Since several. aspirin-like drugs are known to cause acid back-diffusion following intragastric administration, their potency in producing gastric erosions may be related to their concurrent effects on mucosal blood flow.

This work was supported by an M.R.C grant to Professor G.P. Lewis. Prostaglandins were kindly supplied by the Upjohn Company, Kalamazoo.

References

CHVASTA, T E & COOKE, A R. (1972) The effect of several ulcerogenic drugs on the canine gastric mucosal barrier *J. Lab. clin Med.*, 79, 302-315.

MAIN, I H M. & WHITTLE, B.J R. (1973). Gastric mucosal blood flow during pentagastrin- and histamine-stimulated acid secretion in the rat. *Br. J. Pharmac.*, **49**, 534-542

MAIN, I.H.M. & WHITTLE, B J.R (1975). Investigation of the vasodilator and antisecretory role of prostaglandins in the rat gastric mucosa by use of non-steroidal anti-inflammatory drugs *Br. J. Pharmac.*, **53**, 217-224

SEMPLE, P.F & RUSSELL, R I. (1975) Role of bile acids in the pathogenesis of aspirin-induced gastric mucosal haemorrhage in rats. *Gastroenterology*, **68**, 67-70.

WHITTLE, B.J.R. (1975) Gastric acidity and the mechanisms by which prostaglandins prevent indomethacin-induced gastric erosions. *Br J. Pharmac.* **55**, 242-243P

The pro-inflammatory activity of E-, A-, D- and F-type prostaglandins and analogues 16, 16-dimethyl-PGE$_2$ and (15S)-15-methyl-PGE$_2$ in rabbit skin; the relationship between potentiation of plasma exudation and local blood flow changes

T J. WILLIAMS (introduced by G.P.LEWIS)

Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons of England, London WC2A 3PN

Potentiation of inflammatory exudation by locally-injected prostaglandins has previously been described in guinea-pigs (Williams & Morley, 1973) and in rats (Moncada, Ferreira & Vane, 1973; Thomas & West, 1973). It was suggested (Williams & Morley, 1973) that this potentiation may be a consequence of the vasodilator activity of prostaglandins (PGs). This possibility has been investigated using a technique for the simultaneous measurement of local plasma exudation and blood flow changes in rabbit skin (Williams, 1975). The technique consists of intravenous injection of [^{131}I]-albumin, followed by intradermal injections of inflammatory agents mixed with ^{133}Xe in saline. After a fixed interval the animal is killed, skinned and punched-out lesions counted in a γ-counter. The ^{131}I counts then give a measure of plasma exudation and $\log_e$ ^{133}Xe counts are inversely proportional to local blood flow. Using this technique it was found that intradermal injections of prostaglandins alone produced insignificant plasma exudation, as in the guinea-pig, but that addition of PGE$_1$ or PGE$_2$ to bradykinin or histamine before i.d. injection produced marked potentiation, in doses of prostaglandin down to a few nanograms PGE$_1$ and PGE$_2$ had similar potentiating potency, as did the analogues 16, 16-dimethyl PGE$_2$ and (15S)-15-methyl-PGE$_2$. PGA$_1$ and PGA$_2$ showed less potentiating activity Potentiation was produced by PGD$_2$ and PGF$_{2\alpha}$ but only at high doses (1 μg/dose). The evidence linking potentiation with vasodilatation is as follows. Firstly, the ranking order of the exudation potentiating activity of the prostaglandins studied, correlated with their vasodilating potency. Secondly, other vasodilators, e.g. adenosine diphosphate (1 μg/dose), produced significant potentiation of histamine responses. Thirdly, PGEs, in spite of their lability, produced prolonged dilatation in skin, and when histamine was administered as a supra-injection (i.d.) 30 min after PGE$_2$ injection, potentiation was still in parallel with the observed increased blood flow. This was also the case with the more persistent effects of the methylated analogues. Fourthly, addition of vasoconstrictors, e.g. angiotensin II, to histamine/prostaglandin mixtures before intradermal injection reduced local blood flow and reduced exudation potentiation in parallel.

Thus it would appear that in rabbit skin, vasodilatation (perhaps by increasing transmural hydrostatic pressure gradients and vessel wall area), may be responsible for the potentiation of inflammatory exudation produced by prostaglandins.

This work was supported by an M R C. Grant to Professor G P Lewis. Prostaglandins were kindly supplied by the Upjohn Company, Kalamazoo

References

MONCADA, S., FERREIRA, S.H. & VANE, J.R. (1973). Prostaglandins, aspirin-like drugs and the oedema of inflammation. *Nature, Lond* , 246, 217-218.

THOMAS, G. & WEST, G.B. (1973). Prostaglandins as regulators of bradykinin responses *J. Pharm. Pharmac.*, 25, 747-748.

WILLIAMS, T.J. & MORLEY, J. (1973). Prostaglandins as potentiators of increased vascular permeability in inflammation. *Nature, Lond* , 246, 215-217.

WILLIAMS, T.J. (1975). Simultaneous measurement of local plasma exudation and blood flow changes induced by intradermal injection of vasoactive substances, using ^{131}I-albumin and ^{133}Xe. *J Physiol, Lond* , (in press).

The effects of anti-inflammatory steroids on levels of prostaglandin in adipose tissue *in vitro*

J. CHANG*, G.P. LEWIS & PRISCILLA J. PIPER

Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons, London WC2A 3PN

Lewis and Piper (1975) have postulated that anti-inflammatory steroids inhibit the prostaglandin mediated vasodilatation accompanying lipolysis in adipose tissue by preventing the release of prostaglandin (PG). This hypothesis has now been tested further using an *in vitro* system.

Female New Zealand white rabbits were killed and the epigastric fat depots removed. The fat tissue was rinsed in Krebs medium and divided into portions (5g), chopped into pieces about 2 mm^3 and washed three times with Krebs medium. The chopped fat was placed in fresh Krebs medium to a final volume of 10 ml. Each portion was pre-incubated for 20 min at room temperature with or without the anti-inflammatory drug before the addition of ACTH$_{1\text{-}24}$ (0.1 μg/ml) and then incubated for a further 120 minutes. After incubation, the Krebs medium was separated by filtration; a fresh volume was added to the chopped fat and again filtered. The two filtrates were combined. The chopped fat was placed in ice-cold ethanol (10 ml) and both the fat and supernatant extracted into ethyl acetate. Since only small amounts of PG were released on incubation with ACTH$_{1\text{-}24}$, radioimmunoassay (Hennam, Johnson, Newton & Collins, 1974) using an antiserum which cross-reacted 100% with PGE$_2$ and 44% with PGE$_1$, was used to measure the PG content.

In order to provide further evidence of identification of the PG, pooled ACTH stimulated extracts were subjected to thin layer chromatography in the AII system. The plate was then divided into 1 cm zones, silica gel scraped off and PGs estimated on rat stomach strip, chick rectum and rat colon. The activity recovered from the plates corresponded to PGE$_2$.

The release of glycerol, measured by the method of Eggstein (1966), was used to monitor the lipolytic action of ACTH. The increase of glycerol levels during ACTH stimulation was not affected by the doses of indomethacin and corticosteroids used, indicating that the anti-inflammatory drugs did not reduce lipolysis.

When the chopped fat tissue was incubated in Krebs medium alone a small amount of PGE$_2$ (measured as ng/5g fat tissue) appeared in the fat sediment (5.4 ± 0.4) and in the supernatant (4.9 ± 0.5)

After stimulation with ACTH there was a statistically significant increase in the PG content of the fat (13.4 ± 0.7) and of the supernatant (17.0 ± 1.3). When incubated in the presence of indomethacin (1 μg/ml), ACTH failed to cause an increase either in the fat (1.5 ± 0.3) or supernatant (2.3 ± 0.5), the PG levels remaining below control values. On the other hand, in the presence of the corticosteroid, betamethasone (10 μg/ml) the content of PGE$_2$ in the supernatant was lower (5.8 ± 0.8) whilst that in the fat was higher (24.4 ± 1.5) than with ACTH alone. Hydrocortisone (10 μg/ml) produced the same effect.

Thus whereas a non-steroid anti-inflammatory agent, indomethacin, reduced the total amount of PG formed during lipolysis, anti-inflammatory steroids did not reduce the total but increased the tissue/supernatant ratio. These results support the hypothesis that in rabbit adipose tissue corticosteroids inhibit the release of prostaglandins but not their synthesis.

J.C. is an M R.C. research student

References

EGGSTEIN, M. (1966). A new determination of neutral fat in blood serum and tissue. II. Reliability of the method, other neutral fat determinations, normal values for triglycerides and glycerine in human blood *Klin. Wschr.*, 44, 267-273.

HENNAM, J.F., JOHNSON, D.A., NEWTON, J.R & COLLINS, W.P (1974) Radioimmunoassay of prostaglandin $F_{2\alpha}$ in peripheral venous plasma from men and women. *Prostaglandins*, 5, 531-542.

LEWIS, G.P & PIPER, PRISCILLA, J. (1975). Inhibition of release of prostaglandins as an explanation of some of the actions of anti-inflammatory corticosteroids. *Nature, Lond.*, 254, 308-311.

Effects of steroid hormones on tissue levels of prostaglandin 15-hydroxydehydrogenase in the rat

G.J. BLACKWELL & R.J. FLOWER

Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

Blackwell, Flower & Vane (1975) showed that prostaglandin 15-hydroxydehydrogenase (PGDH) —the enzyme catalysing the initial step in prostaglandin metabolism—has a short life within the cell, and suggested that the enzyme might be under hormonal control many metabolic processes and there is already some evidence that steroids, after PGDH levels in the pregnant rabbit (Bedwani & Marley, 1975; Sun & Armour, 1974). We have now studied the tissue activity of PGDH in rats in which steroid hormone levels were altered by adrenalectomy, ovariectomy or pregnancy.

PGDH activity was estimated in the high speed supernatants of kidneys (and sometimes in lungs) by measuring the conversion of [^{3}H]-PGE$_2$ to its 15-keto derivative as previously described (Blackwell, Flower, Parsons & Vane 1975). Metabolism in the particle-free fractions of kidneys from adrenalectomized rats was greatly (140%) increased above control levels (see Table 1); however, after hydrocortisone hemisuccinate (two doses of 5 mg i.p., 8 h apart) PGDH activity was returned to control levels or below. A synthetic glucocorticoid, dexamethasone (two doses of 1 mg i.p., 8 h apart) also reduced the tissue levels of PGDH in control rats by about 40%.

Metabolism in high speed fractions of ovariectomized rat kidneys was again higher (116%) than in the control group. Administration of oestradiol-17β (1 mg each day for 3 days) reduced metabolism to 17% of control levels whilst progesterone (same dose) had only a very slight inhibitory effect (< 5%).

None of the exogenous steroids had any direct effect on enzyme activity *in vitro*.

Metabolism of PGE$_2$ in the lungs and kidneys of pregnant rats varied; for example in well advanced pregnancy (day 18) metabolism by lung was higher than in the ovariectomized controls (185%). However, in rats during parturition the enzyme levels were extremely low, less than 3% of control levels. Similar effects were seen on kidney PGDH levels.

These results suggest that exogenous or endogenous steroid hormones can greatly modify PGDH levels. In particular the profound

Table 1 Effect of steroid hormones on tissue levels of PGDH in the rat

Tissue source	*Treatment*	*PGE$_2$ oxidized* fmol mg^{-1} min^{-1} (mean ± s.e. mean)	
Kidney	Control (♂)	594.4 ± 9.5	(n = 5)
	Adrenalectomized	835.5 ± 5.5	(n = 3)
	Control + hydrocortisone	525.0 ± 3.1	(n = 3)
	Adrenalectomized + hydrocortisone	486.7 ± 4.1	(n = 3)
	Control + dexamethasone	348.1 ± 6.6	(n = 5)
	Control (♀)	609.6 ± 82.2	(n = 3)
	Ovariectomized	704.4 ± 25.6	(n = 5)
	Ovariectomized + progesterone	583.4 ± 19.0	(n = 5)
	Ovariectomized + oestradiol 17β	103.2 ± 5.4	(n = 5)
Lung	Control (♀-ovariectomized)	914.4 ± 121.57	(n = 3)
	Pregnant (18 day)	1699.6 ± 58.8	(n = 3)
	Pregnant (Parturition)	24.8 ± 6.6	(n = 3)

depression of PGDH levels occurring during parturition—which could perhaps be due to the oestrogen surge occurring about this time—could be a controlling factor in determining the rise in prostaglandin activity during parturition.

References

BEDWANI, J.R. & MARLEY, P B. (1975). Enhanced inactivation of prostaglandin E$_2$ by the rabbit lung during pregnancy or progesterone treatment. *Br. J. Pharmac.*, 53, 547-554.

BLACKWELL, G.J., FLOWER, R.J. & VANE, J.R. (1975). Rapid reduction of prostaglandin 15-hydroxy-dehydrogenase activity in rat tissues after treatment with protein synthesis inhibitors. *Br. J. Pharmac*, 55, 233-238

BLACKWELL, C.J., FLOWER, R.J., PARSONS, M.F. & VANE, J.R. (1975). Factors influencing the turnover of prostaglandin synthetase. *Br. J. Pharmac.*, 53, 467-468P

SUN, F.F. & ARMOUR, S.B. (1974). Prostaglandin 15-hydroxydehydrogenase and Δ 13-reductase levels in the lungs of maternal, fetal and neonatal rabbits. *Prostaglandins*, 7, 327-338.

Formation of prostaglandin endoperoxides and rabbit aorta contracting substance (RCS) by coupling two enzyme systems

S. BUNTING, S. MONCADA*, P. NEEDLEMAN & J.R. VANE

The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

Piper & Vane (1969) detected the release of an additional and labile substance during anaphylaxis in isolated lungs from sensitized guinea-pigs. Because of its activity, they called it 'rabbit aorta contracting substance' or RCS. The half life of RCS was less than 2 minutes. Thromboxane A$_2$ (TxA2) has a half life of 30 s, induces platelet aggregation and contracts rabbit aorta; it is thought to be RCS (Samuelsson, 1975). TxA$_2$ is generated from prostaglandin (PG) G$_2$ or PGH$_2$ by tissues such as lungs or platelets (Samuelsson, 1975).

We have used washed platelets obtained from fresh citrated horse blood to show that conversion of PGG_2 to TxA_2 is enzymic The platelets were disrupted by freezing and thawing three times. The lysate was centrifuged at 5000 *g* for 50 min and the supernatant recentrifuged at 100,000 *g* for 60 minutes. The resultant microsomal pellet was washed and re-suspended in 2 ml of 100 mM Tris (pH 7 5) buffer.

The biological activity of the products was assayed on a rat stomach strip and rabbit aorta superfused at 10 ml per min with Krebs' solution at 37° C containing a mixture of antagonists (Gilmore, Vane & Wyllie, 1968) plus indomethacin (1 μg/ml) to make the assay more specific.

PGG_2 or PGH_2 contracted rabbit aorta and rat stomach strip in a concentration dependent manner. When PGG_2 or PGH_2 was incubated at 0° C with horse platelet microsomes and immediately tested, the contraction of the rabbit aorta was greatly augmented, the contraction of the rat stomach strip was substantially reduced, or disappeared altogether.

When PGG_2 was incubated with intact platelets, a powerful rabbit aorta contracting substance was also produced Neither boiled platelet microsomes nor the 100,000 *g* supernatant fraction from lysed platelets converted PGG_2 or PGH_2 into a more potent compound.

PGG_2 has a half life in aqueous solution of about 5 min (Hamberg & Samuelsson, 1973), after standing at room temperature in an aqueous solution for 25 min, the intrinsic contractor activity on rabbit aorta disappeared, as well as the ability to generate RCS after incubation with platelet microsomes.

When arachidonate was incubated (without cofactors) with the microsomal preparation of ram seminal vesicles prepared according to Takeguchi, Kotina & Sih (1971), a product was formed which behaved like PGG_2 or H_2. It contracted rat stomach strip and rabbit aorta and had a half life of 3-5 minutes. It was converted to a more potent RCS-like substance ($t_{1/2}$ = 30 s) by incubation with platelet microsomes. Thus, we consider seminal vesicle microsomes plus arachidonate as an endoperoxide generating system (i.e. cyclo-oxygenase) and platelet microsomes plus PGG_2 as an RCS (TxA_2) generating system (thromboxane synthetase).

The use of these two enzyme systems, either alone or in combination, offers a relatively simple source of endoperoxides and TxA_2 which are extremely unstable materials otherwise difficult to obtain. The transformation of endoperoxides to TxA_2 could be of importance in physiological or pathophysiological conditions.

References

GILMORE, N., VANE, J.R. & WYLLIE, H.J. (1968). Prostaglandins released by the spleen. *Nature, Lond.*, **218**, 1135-1140.

HAMBERG, M & SAMUELSSON, B (1973) Detection and isolation of an endoperoxide intermediate in prostaglandin biosynthesis. *Proc natn. Acad Sci., U.S.A.*, **70**, 899-903.

PIPER, P.J. & VANE, J.R. (1969). The release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. *Nature, Lond*, **223**, 29-35.

SAMUELSSON, B. (1975). Opening address Second International Prostaglandin Conference, Florence

TAKEGUCHI, C., KOTINA, E & SIH, C J (1971). Mechanism of prostaglandin biosynthesis. I Characterization and assay of bovine prostaglandin synthetase. *Biochemistry*, **10**, 2372-2376.

Chemotactic activity of solutions of prostaglandin E_1

A.W. FORD-HUTCHINSON,* M.J.H. SMITH & J.R. WALKER

Department of Biochemical Pharmacology, King's College Hospital Medical School, London SE5 8RX

Prostaglandin E_1 but not other prostaglandins (A_1, E_2, $F_{2\alpha}$) has been reported to possess chemotactic activity against rabbit polymorphonuclear leucocytes *in vitro*. The earlier observations were obtained using a concentration of 1 μg/ml (Kaley & Weiner, 1971, McCall & Youlten, 1973) but more recently it has been claimed that the prostaglandin is chemotactic at concentrations down to 10 ng/ml (Higgs, McCall & Youlten, 1975). This finding is of much more relevance to inflammation *in vivo* since it occurred at concentrations below those of the total prostaglandins found in the carrageenin-induced air bleb in the rat (McCall & Youlten, 1974).

Other workers (Turner, Campbell & Lynn, 1975) have failed to detect any chemotactic activity of various prostaglandins, including E_1, towards human polymorphonuclear leucocytes *in vitro* even at concentrations as high as 100 μg/ml. Furthermore the local injection of prostaglandin E_1 into areas of human and rat skin does not cause

Table 1 Chemotactic activity of prostaglandin E_1 (1 μg/ml)

Fresh solution		*Solution after 12 h*	
Random migration	*Directed migration*	*Random migration*	*Directed migration*
62.0 ± 3.5	61.7 ± 5.2	49.8 ± 0.7	96.8 ± 15.2*
17.3 ± 2.3	17.4 ± 0.7	27.4 ± 1.0	47.0 ± 1.1*
29.9 ± 2.8	33.9 ± 7.1	65.9 ± 6.1	182.8 ± 28.6*
125.9 ± 12.0	119.5 ± 16.4	17.4 ± 1.9	30.5 ± 2.0*
73.9 ± 1.3	73.5 ± 1.2	34.4 ± 1.4	45.8 ± 5.8

Results given as number of cells per high power field, each figure being the mean ± s.d. of three chambers For the measurement of random migration PGE_1 was placed in both compartments of the Boyden chambers; for directed migration (chemotaxis) it was present in the lower chamber only

* $P < 0.05$

an increased emigration of leucocytes (Søndergaard & Wolf-Jürgensen, 1972; Arora, Lahiri & Sanyal, 1970).

We have therefore studied the effect of solutions of prostaglandin E_1 on the directed migration of rat polymorphonuclear leucocytes using the Boyden chamber technique described previously (Walker, Smith, Ford-Hutchinson & Billimoria, 1975). The results (Table 1) show that when a 1 μg/ml solution of Prostaglandin E_1 in a suitable aqueous medium, e.g. Medium 199 or Hanks, is tested immediately no chemotactic activity is detectable When the aqueous solution has been allowed to stand either at $-20°$ or at room temperature for 24 h then chemotactic activity appears. It is concluded that the material responsible for this activity is not prostaglandin E_1 itself but some product formed by chemical changes in the aqueous media.

This work was supported by grants from the King's College Hospital and Medical School Research Committee and the National Research Development Corporation

References

ARORA, S., LAHIRI, P K. & SANYAL, R.K. (1970) The role of prostaglandin E_1 in inflammatory process in the rat *Int. Arch. Allergy*, 39, 186-191.

HIGGS, G A., McCALL, E. & YOULTEN, L.J F. (1975) A chemotactic role for prostaglandins released from polymorphonuclear leucocytes during phagocytosis. *Br. J. Pharmac.*, 53, 539-546.

KALEY, G & WEINER, R. (1971). Prostaglandin E_1, a potential mediator of the inflammatory response *Ann N Y Acad Sci.*, 180, 338-350.

McCALL, E & YOULTEN, L.J.F (1973) Prostaglandin E_1 synthesis by phagocytosing rabbit polymorphonuclear leucocytes, its inhibition by indomethacin and its role in chemotaxis. *J. Physiol., Lond*, 234, 98-100P.

McCALL, E. & YOULTEN, L.J.F. (1974) The effect of indomethacin and depletion of complement on cell migration and prostaglandin levels in carrageenin-induced air bleb inflammation. *Br J. Pharmac.*, 52, 452P.

SØNDERGAARD, J. & WOLF-JÜRGENSEN, P. (1972) Cellular exudate of human cutaneous inflammation induced by prostaglandin E_1 and $F_{2\alpha}$ *Acta Derm., (Stockholm)*, 52, 361-364

TURNER, S.R., CAMPBELL, J A. & LYNN, W.S. (1975) Polymorphonuclear leukocyte chemotaxis toward oxidized lipid components of cell membranes. *J exp Med.*, 141, 1437-1441.

WALKER, J.R., SMITH, M.J.H., FORD-HUTCHINSON, A.W. & BILLIMORIA, F J. (1975) Mode of action of an anti-inflammatory fraction from normal human plasma. *Nature, Lond.*, 254, 444-446.

Inhibition of phagocytosis by mepacrine

DOROTHEA J. BAKER, D.G. TRIST* & M. WEATHERALL

Wellcome Research Laboratories, Beckenham, Kent

When human or horse plasma containing leucocytes is passed through a column of glass wool, most of the granulocytes, monocytes and platelets are trapped but most of the lymphocytes are not. The number of cells sticking can be measured by counting the cells in the plasma before and after it has passed through the column. In conditions which will be described, 43.0 ± 4.6% (mean ± s.e. mean, $n = 10$) of the leucocytes applied are recovered When mepacrine hydrochloride is added to the plasma, fewer cells stick. Concentrations below 10^{-4} M have little effect· with 10^{-3} M in plasma 63.0 ± 4.8% ($n = 8$) of the cells are recovered

Granulocytes which have been adsorbed on glass wool can be recovered by washing with ice-cold sodium citrate buffer. Such cells have been resuspended in a physiological salt solution and maintained at 37° C in order to measure their potassium content and turnover (Baker, Trist & Weatherall, 1975). When mepacrine (10^{-5} M to 10^{-3} M) is added, K^+ entry to the cells is reduced and the total cell potassium diminishes. The EC_{50} for inhibition of potassium entry by mepacrine in these experiments is about 0.6 mM

When yeast or latex particles are added to a suspension of granulocytes, phagocytosis occurs promptly, as can be shown by electron micrographs. Potassium entry is sometimes increased in the first 10-15 minutes. After 30 min or longer, it is reduced significantly below control values (Dunham, Goldstein & Weissmann, 1974). In the presence of mepacrine (10^{-5} to 10^{-4} M) potassium entry is reduced more rapidly than in the absence of particles. Pseudopodia are not formed on the surface of the granulocytes, and phagocytosis is reduced or absent.

The mechanism by which mepacrine alters the stickiness of cells and prevents uptake of particles and of potassium is not known. Chloroquine acts similarly but is less potent. Chlorpromazine has been shown to inhibit K^+ entry to mononuclear cells and to prevent phagocytosis by monocytes (Ødegaard, 1975). In our experiments it is comparable in potency to mepacrine in inhibiting K^+ entry to polymorphs.

References

BAKER, D.J., TRIST, D.G. & WEATHERALL, M. (1975). *J. Physiol., Lond*, (Demonstration to Physiological Society November 1975).

DUNHAM, P B., GOLDSTEIN, I.M. & WEISSMANN, G (1974). *J. cell Biol.*, 63, 215-226.

ØDEGAARD, A (1975). *Acta path microbiol. scand.* Sect C., 83, 59-73.

Inhibition by antigen and by histamine antagonists of the uptake of histamine by isolated human leucocytes

E.S.K. ASSEM

Department of Pharmacology, University College London

Antigen is known to release histamine from sensitized mast cells and basophil leucocytes, but its possible effect on the reverse process, i.e. the incorporation of histamine, has not been investigated The work to be described deals with this point, using [^{14}C]-ring-labelled histamine (dihydrochloride or phosphate), and the effect of various histamine antagonists, exemplified by mepyramine (an H_1-receptor antagonist, Ash & Schild, 1966), and metiamide, an H_2-receptor antagonist (Black, Duncan, Emmett, Hesselbo, Parsons & Wyllie, 1973).

Two types of human leucocyte preparations were used. 1. Whole leucocyte population (WLP) from patients with allergic airways disease (asthma and rhinitis) due to *Aspergillus fumigatus* or the house-dust mite *Dermatophagoides pteronyssinus*, and from which histamine could be released with these antigens, and with anti-IgE. 2. Isolated blood lymphocytes from tuberculin-sensitive patients.

Incubation of WLP with either the specific antigen or anti-IgE inhibited the uptake of labelled histamine (ULH), the inhibition being related to antigen concentration and to incubation time, reaching a maximum in 30 min (which is slower than histamine release), and persisting after removing the antigen and washing the cells Calcium lack, which inhibited histamine release, abolished the inhibition of ULH.

Mepyramine and metiamide equally inhibited ULH in absence of antigen, but metiamide seemed to have a greater adding effect on the antigen-induced inhibition of ULH (Figure 1).

Tuberculin-sensitized lymphocytes previously incubated with tuberculin for four days showed an *increase* in ULH, which was blocked by metiamide, and to a smaller extent by mepyramine, suggesting the presence of H_2 receptors on lymphocytes. This finding, together with the inhibition of ULH by WLP, though requiring an extension of control studies with drugs other than antihistamines, provide further support for the presence of histamine receptors on human blood leucocytes participating in different allergic reactions, as suggested by a number of authors including Lichtenstein and his collaborators (Plaut, Lichtenstein & Henney, 1973; Lichtenstein & Gillespie, 1973, 1975).

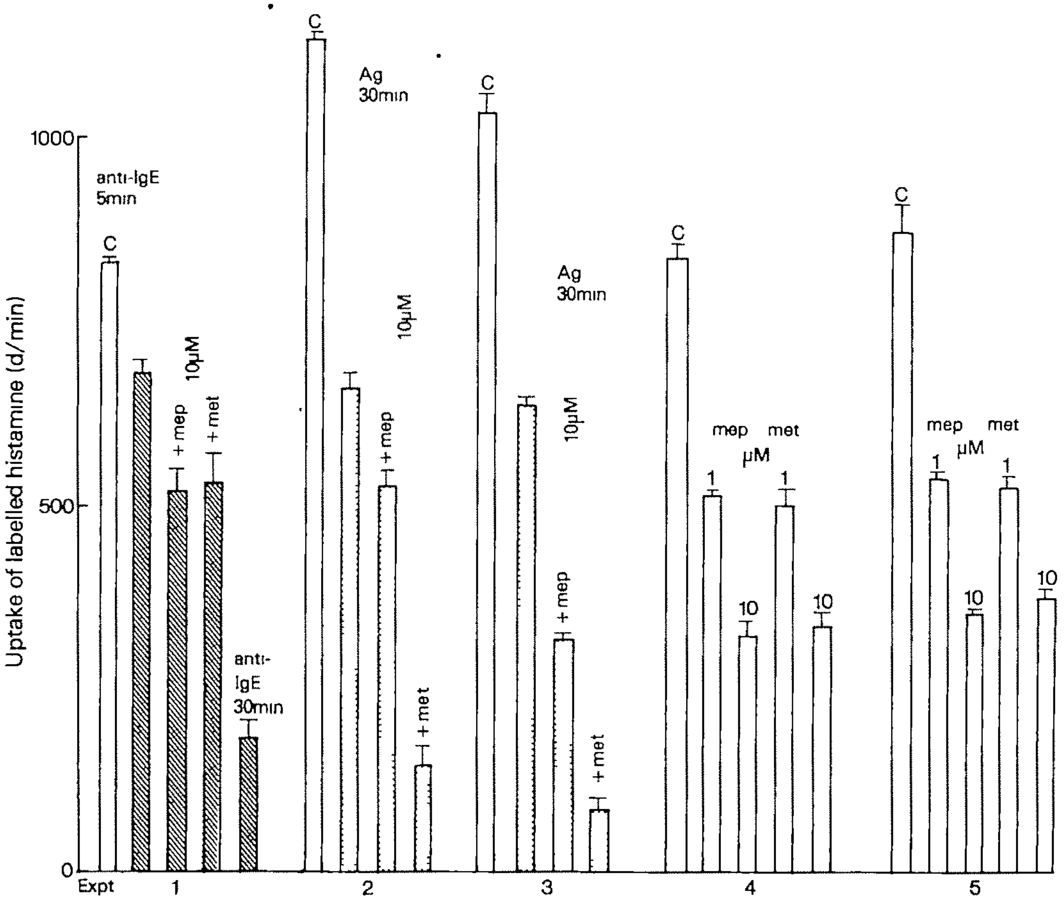


Figure 1 Inhibition of the uptake of labelled histamine by isolated human leucocytes, in five different experiments. Experiments 1, 2 and 3 show the inhibition by anti-IgE, and by the specific allergens, *Aspergillus fumigatus* and house-dust mite extracts, respectively in three different allergic patients, with and without the addition of the histamine antagonists, mepyramine (mep) metiamide (met), 5 min before the simultaneous addition of [^{14}C]-labelled histamine and the allergen. Experiments 4 and 5 show the effects of these two drugs in absence of the allergen (or anti-IgE) challenge (the drugs were added 5 min before the labelled histamine; the incubation with the latter was 30 min), in two of the three patients C = control aliquots The counts of labelled histamine shown represent a mean ± s.e. mean of triplicate aliquots (10^7 cells each) for each treatment; all aliquots obtained from the same venous blood sample.

References

ASH, A.S F & SCHILD, H.O. (1966). Receptors mediating some actions of histamine. *Br. J. Pharmac. Chemother.*, 27, 427-439.

BLACK, J.W., DUNCAN, W.A.M., EMMETT, J C., HESSELBO, M.E., PARSONS, M.E. & WYLLIE, J.H. (1973). Metiamide—an orally active histamine antagonist. *Agents and Actions*, **3**, 133-137.

LICHTENSTEIN, L.M & GILLESPIE, E. (1973). Inhibition of histamine release by histamine controlled by H_2 receptor. *Nature, Lond.*, **244**, 287-288.

LICHTENSTEIN, L.M. & GILLESPIE, E (1975). The effects of the H_1 and H_2 antihistamines on 'allergic' histamine release and its inhibition by histamine. *J. Pharmac exp. Ther.*, **192**, 411-440.

PLAUT, M., LICHTENSTEIN, L.M & HENNEY, C.S. (1973). Increase in histamine receptors on thymus-derived effector lymphocytes during the primary immune response to alloantigens. *Nature, Lond.*, **244**, 285-286.

The nature of the bradykinin inactivating system in isolated lungs

Y.S. BAKHLE

Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons of England, Lincoln's Inn Fields, London WC2A 3PN

Bradykinin is extensively inactivated in the pulmonary circulation *in vivo* and in isolated lungs (for references, see Bakhle & Vane, 1974). Some of this inactivation is undoubtedly due to the action of a dipeptidyl carboxypeptidase (converting enzyme, kininase II, EC.3 4.15.1) which hydrolyses both bradykinin and angiotensin I. However, there are other peptidases (amino-, endo- or carboxy-) capable of inactivating bradykinin, as the cleavage of any peptide bond causes 99% biological inactivation (Suzuki, Abiko, Endo, Kameyama, Sasaki & Nabeshima, 1969) and we have tried to assess the contribution of peptidases other than converting enzyme to the overall inactivation of bradykinin in isolated perfused lungs by studying the fate of two analogues of bradykinin in this system.

The analogues used were 7-β homo Pro-bradykinin (7 HBK) and 8-β homo Phe-bradykinin (8 HBK) (Ondetti & Engel, 1975) and their inactivation in rat and guinea-pig isolated lungs was measured by bioassay using either the guinea-pig ileum or the cat terminal ileum (Alabaster & Bakhle, 1972) The results given are the means of at least six preparations. In rat lungs all the peptides were extensively inactivated and very little activity survived a single passage through the pulmonary circulation—bradykinin, 2.8 ± 0.8%, 7 HBK 10.6 ± 1 6%; 8 HBK 3.8 ± 1.2%. However, in guinea-pig lungs, whereas only a tenth of bradykinin (9.5 ± 1.0%) and 8 HBK (10.0 ± 1.2%) activity survived passage through the pulmonary circulation, nearly half of the activity of infusions of 7 HBK (42.0 ± 6.0%) emerged from the lung. The *Bothrops* nonapeptide, Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, inhibits the pulmonary inactivation of bradykinin (Greene, Camargo, Krieger, Stewart & Ferreira, 1972; Engel, Schaeffer, Gold & Rubin, 1972) by its inhibition of converting enzyme (Bakhle, 1974, Dorer, Kahn, Lentz, Levine & Skeggs, 1974, Das & Soffer, 1975). Infusion of the nonapeptide (100 ng/ml) through the pulmonary circulation protected bradykinin and 8 HBK from inactivation in guinea-pig lung, the percent survival increasing to 58.4 ± 4.1 and 55.8± 4.4 respectively whereas the inactivation of 7 HBK was not significantly affected. In rat lung the survival of 7 HBK was again unaffected by the nonapeptide whereas that of bradykinin and 8 HBK was increased to 12.2 ± 1.2% and 13.6 ± 2.8% respectively.

As the inactivation of 7 HBK is relatively resistant to the nonapeptide inhibitor we conclude that this analogue is not a substrate for converting enzyme, whereas 8 HBK and bradykinin are both substrates. This is compatible with the finding that 7 HBK was a more potent depressor agent than 8 HBK *in vivo* and whereas the hypotensive actions of 8 HBK and bradykinin were potentiated, that of 7 HBK was not (Ondetti & Engel, 1975). Our results also show that in isolated guinea-pig lung almost half of the observed bradykininase activity is converting enzyme-like, whereas in rat lung most of the bradykininase activity is not converting enzyme-like. This may explain results suggesting that the conversion of angiotensin I in rat lung is less than in the lungs of other species (Bakhle, Reynard & Vane, 1969; Kreye & Gross, 1971).

I thank Dr M A Ondetti (Squibb Institute for Medical Research, Princeton, N.J.) for the analogues; Sandoz (Basel) for a gift of bradykinin and the M.R.C. for support

References

ALABASTER, V A. & BAKHLE, Y S (1972). The inactivation of bradykinin in the pulmonary circulation of isolated lung. *Br. J. Pharmac.*, **45**, 299-309.

BAKHLE, Y S (1974) Converting enzyme, *in vitro* measurement and properties. In *Handb. exp. Pharmac.* Vol. 37, ed. Page, I.H. & Bumpus, F.M., pp. 41-80. Berlin and Heidelberg Springer-Verlag.

BAKHLE, Y.S., REYNARD, A.M. & VANE, J.R. (1969). Metabolism of the angiotensins in isolated perfused tissues. *Nature, Lond*, **222**, 956-959.

BAKHLE, Y.S. & VANE, J.R. (1974). Pharmacokinetic function of the pulmonary circulation. *Physiol. Rev.*, **54**, 1007-1045

DAS, M. & SOFFER, R.L. (1975) Pulmonary angiotensin converting enzyme structural and catalytic properties. *J. biol. Chem.*, **250**, 6762-6768.

DORER, F.E., KAHN, J.R., LENTZ, K.E., LEVINE, M. & SKEGGS, L T. (1974). Hydrolysis of bradykinin by angiotensin converting enzyme. *Circ. Res*, **34**, 824-827.

ENGEL, S L , SCHAEFFER, T.R , GOLD, B.I. & RUBIN, B. (1972). Inhibition of pressor effects of angiotensin I and augmentation of depressor effects of bradykinin by synthetic peptides. *Proc. Soc. exptl. Biol Med.*, **140**, 240-244

GREENE, L.J., CAMARGO, A C., KRIEGER, E.M , STEWART, J.M. & FERREIRA, S.H (1972) Inhibition of the conversion of angiotensin I to II and potentiation of bradykinin by small peptides present in *Bothrops jararaca* venom. *Circ. Res.*, **30 & 31**, Suppl. **II**, 62-71.

KREYE, V A.W. & GROSS, F. (1971). Conversion of angiotensin I to angiotensin II in peripheral vascular beds of the rat. *Am. J. Physiol*, **220**, 1294-1296

ONDETTI, M A. & ENGEL, S.L. (1975). Bradykinin analogs containing β-Homo-amino acids. *J. med. Chem.*, **18**, 761-763.

SUZUKI, K , ABIKO, T., ENDO, N., KAMEYAMA, T., SASAKI, K. & NABESHIMA, J (1969). Biologically active synthetic fragments of bradykinin. *Jap. J. Pharmac.*, **19**, 325-327

Effects of some fatty acids on gallbladder function

I.K.M. MORTON, G.E ROSE*, S H. SAVERYMUTTU & J.R WOOD

Department of Pharmacology, King's College, London

Prostaglandins are known to have actions both on fluid transport and on smooth muscle of the gallbladder (Morton, Saverymuttu & Wood, 1974). We have now investigated the effects of other fatty acids on gallbladder function.

In vitro experiments with intact gallbladders taken from male guinea-pigs showed that arachidonic acid, a known prostaglandin precursor, inhibited fluid transport, in a dose-dependent manner, within 30 min of serosal application in the concentration range 10-200 μM. Inhibition was preceded by a transient phase of enhanced fluid loss that was concurrent with a rise in intraluminal pressure; these last two effects probably being related (Morton, Saverymuttu & Wood, 1975).

Since these actions of arachidonic acid are seen with somewhat lower concentrations of prostaglandins of the E or F series (Morton, *et al.*, 1974) the possibility of conversion must be considered Indomethacin (1 μM) was shown to antagonize the actions of arachidonic acid but, in contrast, to have little effect on those of ricinoleic acid, a fatty acid not thought to be converted to prostaglandin, although found here to have properties and potency similar to arachidonic acid when tested on the gallbladder.

These experiments suggest that, although arachidonic acid and ricinoleic acid have similar actions on gallbladder fluid transport and smooth muscle, the former owes some of its potency to conversion to prostaglandin. This is in agreement with the fining that the spasmogenic action of arachidonic acid on gallbladder strips is antagonized by indomethacin (Andersson, Hedner & Persson, 1974).

In view of similarities in the transport systems, these findings may also throw light on gastrointestinal function. It is well known, for instance, that ricinoleic acid has a pronounced cathartic action, although it is uncertain whether this is due to effects on fluid transport or on smooth muscle (Fingl, 1970).

References

ANDERSSON, K E., HEDNER, P. & PERSSON, C.G.A. (1974). Differentiation of the contractile effects of prostaglandin E_2 and the C-terminal octapeptide of cholecystokinin in isolated guinea-pig gallbladder. *Acta physiol. scand.*, **90**, 657-663.

FINGL, E. (1970). Cathartics and laxatives. In *The Pharmacological Basis of Therapeutics*, ed Goodman, L.S , Gilman, A., pp. 1020-1031. London: Collier-Macmillan.

MORTON, I K.M., SAVERYMUTTU, S.H. & WOOD, J.R. (1974). Inhibition by prostaglandins of fluid transport in the isolated gallbladder of the guinea-pig. *Br. J. Pharmac.*, **50**, 460P.

MORTON, I.K.M , SAVERYMUTTU, S.H & WOOD, J.R (1975) Enhanced fluid transfer by the gallbladder following intravesicular pressure changes induced by spasmogens. *J. Physiol., Lond*, (in press).

Species variation in motor transmission to the retractor penis muscle

N. AMBACHE & S.W. KILLICK*

Medical Research Council, Department of Physiology, Royal College of Surgeons of England, London WC2A 3PN

The contractions elicited by transmural stimulation (1-16 pulses; 0.2 ms, 10 Hz) of isolated retractor penis (RP) strips from dogs, pigs, sheep and horses were abolished by tetrodotoxin (1.6 μM) and were therefore neurogenic.

Although, in all species, motor responses to noradrenaline (0.6-3 μM) or tyramine (12-58 μM) were abolished by phentolamine (5.3 μM) or phenoxybenzamine (2.9 μM), the susceptibility of the neurogenic contractions to α-blockade was subject to species variation. In dog RP, the whole of the motor response was promptly abolished by phentolamine or phenoxybenzamine. But in pig RP phentolamine never abolished and only slightly reduced these responses (by < 5% with single pulses and < 34% with 16 pulses), without further reduction on raising the phentolamine concentration to 26.5 μM or on switching to phenoxybenzamine (2.9 μM) for 1 h (Figure 1); or on addition of propranolol (3.4 μM), atropine (2.9 μM), mepyramine (1.25 μM) or dimethyltubocurarine (23 μM).

In pig RP the phentolamine-resistant sympathetic motor transmission was reduced but never completely abolished by bretylium (48 μM) or guanethidine (40 μM), there was a tetrodotoxin-susceptible 10% remnant even after 80 μM guanethidine. Reserpine pretreatment (10 mg kg^{-1} day^{-1} for 3 days) abolished responses to tyramine (105 μM) but not the neurogenic contractions (4-16 pulses) which were wholly phentolamine-resistant.

A considerable phentolamine-resistant component was also found in sheep but not in horse RP.

Thus, whereas in the dog and horse the motor transmission to the retractor penis is wholly adrenergic, as found by previous workers, in the pig and sheep a large proportion of the motor response is unaffected by α-adrenoceptor blockade and appears to resemble the non-adrenergic type of motor transmission found in the vas deferens of many species (Ambache & Zar, 1971, Ambache, Dunk, Verney & Zar, 1972; Euler & Hedqvist, 1975, Jenkins, Marshall & Nasmyth, 1975).

In phenoxybenzamine-treated pig RP, twitch-inhibition by noradrenaline (6 μM) was blocked by propranolol (3.4 μM).

We thank Mr N. Gillespie for valuable help.

References

AMBACHE, N. & ZAR, M. ABOO. (1971) Evidence against adrenergic motor transmission in the guinea-pig vas deferens. *J. Physiol, Lond.*, 216, 359-389

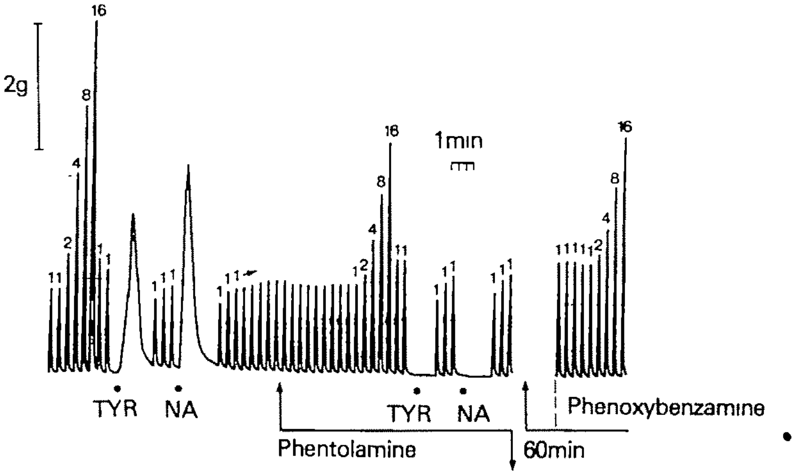


Figure 1 Pig RP. Persistence of a large proportion of the motor responses to 1-16 pulses (0.2 ms, 10 Hz; 1 min intervals; train length indicated by superscripts) in phentolamine (26.5 μM) and then in phenoxybenzamine (2.9 μM for 1 h). At the dots, tyramine (TYR; 26 μM) or noradrenaline (NA, 1.5 μM) for 60 seconds.

AMBACHE, N., DUNK, LINDA P., VERNEY, J & ZAR, M. ABOO. (1972). Inhibition of post-ganglionic motor transmission in vas deferens by indirectly acting sympathomimetic drugs. *J. Physiol, Lond*, 227, 433-456.

EULER, U.S. & HEDQVIST, P. (1975). Evidence for an α- and β_2-receptor mediated inhibition of the twitch response in the guinea-pig vas deferens by noradrenaline. *Acta physiol scand.*, 93, 572-573.

JENKINS, D.A., MARSHALL, I. & NASMYTH, P.A. (1975). Is noradrenaline the motor transmitter in the mouse vas deferens? *J. Physiol., Lond.*, (in press).

Effects of morphine on acetylcholine release from the frog spinal cord

A. NISTRI

Institute of Pharmacology, University of Florence, Italy

Varying reports on the effects of morphine on acetylcholine (ACh) release from the cerebral cortex have been made. For example, in the cat morphine either depresses (Jhamandas, Phillis & Pinsky, 1971) or stimulates (Phillis, Mullin & Pinsky, 1973) ACh release. The opiate action on cortical cholinergic fibres is probably indirect but further experiments are needed to clarify this matter. To this end the frog spinal cord was chosen as an *in vitro* CNS preparation in order to study the effects of bath-applied morphine on spinal root potentials and endogenous ACh release with the method previously reported (Nistri, 1975). The effects of morphine on the ACh content of the frog brain and spinal cord *in vivo* have already been reported (Nistri, Pepeu, Cammelli, Spina & De Bellis, 1974).

The spontaneous ACh release from the frog cords was 7.3 ± 1.06 ng/ml every 10 min (mean ± s.e. mean, $n = 28$). The effects of morphine were variable and related to the concentrations used. Ten minutes after the application of 1 μM morphine spontaneous ACh release was reduced by 29.7 ± 5.5%, a similar decrease was also found in subsequent samples. However, with this dose of morphine a 10 min antidromic ventral root stimulation (1 Hz, 0.1 msec, supramaximal voltage) was accompanied by a 246.8 ± 70.1% increase in ACh output over preceding values whereas in untreated cords similar stimulation yielded a 177.7 ± 50.7% rise The dorsal root potential produced by such stimulation was slightly reduced by 1 μM morphine. All these effects were reversible on washing. Ten minutes after 100 μM morphine was added to the bathing fluid a 142.2 ± 43.2% rise in unstimulated ACh output was seen, the increase persisted in the following samples. However ventral root stimulation could not produce any further rise in ACh output. A small increase in ventral as well as dorsal root potentials was found. Naloxone (100 μM) slightly stimulated the spontaneous ACh release (+ 51.4 ± 15 3%), prevented the decrease in ACh output following 1 μM morphine and reduced the stimulation of ACh release after high doses of morphine.

In the frog spinal cord the motor axon collaterals are cholinergic fibres (Mitchell & Phillis, 1962) which can be directly activated by antidromic ventral root stimulation. Since small concentrations of morphine depressed spontaneous ACh output but failed to reduce the electrically-evoked ACh output, it is suggested that morphine reduced ACh release through an indirect mechanism probably mediated by interneurones. The stimulant action of high concentrations of morphine on spinal ACh release and root potentials might be one of the factors involved in the behavioural excitation seen in frogs after large doses of this opiate.

References

JHAMANDAS, K., PHILLIS, J.W. & PINSKY, C (1971) Effects of narcotic analgesics and antagonists on the *in vivo* release of acetylcholine from the cerebral cortex of the cat. *Br. J. Pharmac.*, 43, 53-66.

MITCHELL, J.F & PHILLIS, J.W. (1962). Cholinergic transmission in the frog spinal cord. *Br J. Pharmac.*, 19, 534-543.

NISTRI, A. (1975). The spinal cord of the frog as an *in vitro* preparation to investigate the acetylcholine output. *J. Physiol., Lond.*, 246, 32-33P.

NISTRI, A, PEPEU, G., CAMMELLI, E., SPINA, L. & DE BELLIS, A.M (1974) Effects of morphine on brain and spinal acetylcholine levels and nociceptive threshold in the frog *Brain Res*, 80, 199-209.

PHILLIS, J W, MULLIN, W.J. & PINSKY, C. (1973). Morphine enhancement of acetylcholine release into the lateral ventricle and from the cerebral cortex of unanaesthetized cats. *Comp. gen. Pharmac.*, 4, 189-200.

The uptake of weak acids and bases into isolated rat superior cervical ganglia in relation to intracellular pH

J. GARTHWAITE (introduced by D.A. BROWN)

Department of Pharmacology, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX

Under conditions where direct measurement is not feasible, intracellular pH (pH_i) may be estimated from the relative concentration of a weak acid or base in the intra- and extra-cellular fluids. Since its introduction by Waddell & Butler (1959), the weak acid 5,5-dimethyl-2,4-oxazolidinedione (DMO) has become the most frequently used marker for cell pH.

Previous measurements of [^{14}C]-DMO distribution in isolated sympathetic ganglia suggested a pH_i of 7.33 at an extra-cellular pH of 7.37 (Brown & Halliwell, 1972). However, the weak base nicotine (measured under conditions where cell depolarization was averted) accumulated in the ganglion to an extent requiring a much more acid environment (pH_i 6.5-6.6). To explain this, a hypothesis of different pH compartments within the cell was advanced, such that a weak base would distribute in accordance with the pH of the most acid compartment and vice versa for a weak acid; on this basis it was suggested that nicotine distribution might reflect the pH of the largest compartment (the cytoplasm) with greater accuracy than a weak acid.

In an attempt to further test this view—and to exclude possible peculiarities in the distribution of nicotine— the uptake of a number of weak bases and acids into isolated rat superior cervical ganglia, incubated in Krebs' solution (pH 7.4) at 25° C, has been measured using methods previously described (Brown & Halliwell, 1972). As shown in Table 1, other bases tended to give pH_i values similar to those of nicotine, in partial confirmation of predictions.

It may also be noted that, because of cell acidity, basic drugs may accumulate in these cells to a much higher concentration than in the surrounding medium. This may have implications for their pharmacology and toxicity.

Supported by a grant from the American Medical Association Education and Research Foundation.

References

BROWN, D.A. & HALLIWELL, J.V. (1972) Intracellular pH in rat isolated superior cervical ganglion in relation to nicotine-depolarization and nicotine-uptake. *Br. J. Pharmac.*, 45, 349-359.

WADDELL, W.J & BUTLER, J.V. (1959). Calculation of intracellular pH from the distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO). Application to skeletal muscle of the dog. *J. clin. Invest.*, 38, 720-729.

Table 1 Characteristics of the uptake of some weak acids and bases into isolated rat sympathetic ganglia

	pKa at 25° C	*Equilibrium time (min)*	*Concentration range (µM)*	*Ci/Co at equilibrium*	*Calculated* pH_i
A Weak bases					
[^{3}H]-Nicotine	8.01	30	0.096-60	6.172 ± 0.116(20)	6.494 ± 0.009
[^{3}H]-Atropine	9.71	90	0.34-100	7.097 ± 0.155(21)	6.539 ± 0 013
[^{14}C]-Morphine	7.94	90	0.35-1000	6.084 ± 0.136(36)	6.507 ± 0.011
[^{14}C]-Procaine*†	8.91	60	2000	5.228 ± 0.287(4)	6.636 ± 0.013
[^{14}C]-Trimethylamine*	9 81	60	1000-2900	3.180 ± 0.131(10)	6.935 ± 0.023
B Weak acids					
[^{14}C]-DMO	6.33	30	110	0.873 ± 0.007(46)	7.311 ± 0.003
[^{14}C]-Phenobarbitone	7.45	30	8.0-3300	2.664 ± 0.051(18)	Ca 8.0

Results given as mean ± s.e. mean. Number of ganglia used indicated in brackets.

* Values given for concentrations which give apparent saturation of binding components.

† Physostigmine (10^{-5} g/ml) was included in the bathing media to prevent metabolism of procaine.

Ci/Co refers to intracellular to extracellular concentration ratio.

Neuromuscular and cardiovascular studies with dimethyl tubocurarine in anaesthetized cats, rhesus monkeys and man

D.J. CHAPPLE, D.H. ENDERBY, R. HUGHES* & J.P. PAYNE

Department of Pharmacology, The Wellcome Research Laboratories, Beckenham, Kent and Research Department of Anaesthetics, Royal College of Surgeons of England, London WC2 and St. Peter's Hospital, London WC2

The pharmacological properties of dimethyl tubocurarine were first described by Collier, Paris & Woolf (1948). Although it was more potent, caused less ganglion blockade and released less histamine than tubocurarine, it never gained popularity because it was difficult then to prepare batches of consistent potency (Mogey & Trevan, 1950). Recently, we found that neuromuscular paralysing doses of dimethyl tubocurarine, unlike those of most non-depolarizing agents, did not cause vagal blockade (Hughes & Chapple, 1975). These interesting properties merit further investigation.

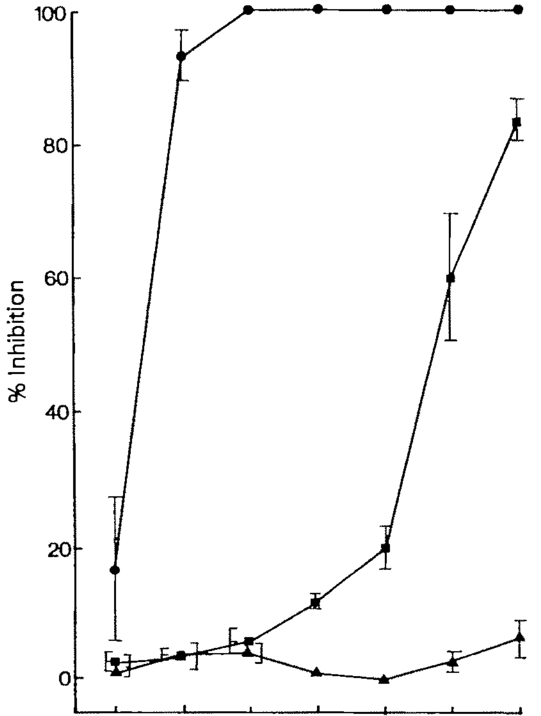


Figure 1 Dose-response curves showing blockade of neuromuscular and autonomic mechanisms by dimethyl tubocurarine given intravenously to cats anaesthetized with chloralose (each point represents the mean of 4 observations). (●) Percentage inhibition of the twitch response of the gastrocnemius muscle to indirect stimulation at 0.1 Hz. (■) Percentage inhibition of the bradycardia response to vagal stimulation at 10-20 Hz per 10 seconds. (▲) Percentage, inhibition of the response of the nicitating membrane to sympathetic nerve stimulation at 10-20 Hz. for 60 seconds. Vertical lines indicate standard errors.

Intravenous doses of dimethyl tubocurarine required for complete neuromuscular paralysis of the twitches of the gastrocnemius muscle were 0.0625 mg/kg for each of 4 cats (Figure 1) and 0.125 mg/kg for 4 monkeys. Vagal blockade only became appreciable at 8-16 times these respective doses; heart rate was unchanged. Sympathetic function was unimpaired in cats (Figure 1) and in monkeys the vasopressor response to carotid occlusion was only reduced (about 10-40%) by supramaximal doses of 0.5-2 mg/kg i.v.; these doses lowered mean blood pressure by 35-46 mmHg in cats and 14-20 mmHg in monkeys. The drug was long acting in cats and monkeys—more than 60 min was required for recovery from complete neuromuscular paralysis.

Preliminary studies, using techniques described previously (Sugai, Hughes & Payne, 1975), were carried out in patients who had given their informed consent before undergoing urological surgery. In 6 anaesthetized patients, intravenous doses of 0.16-0.32 mg/kg dimethyl tubocurarine were required to cause complete neuromuscular paralysis of the twitches of the adductor pollicis muscle, its potency in man corresponded more to that in monkeys than cats. Disturbances in arterial blood pressure and heart rate were minimal. The drug was also long acting in man, more than 60 min was required for recovery, but neuromuscular paralysis was well antagonized by neostigmine.

Thus, dimethyl tubocurarine has desirable clinical properties but the ideal non-depolarizing neuromuscular blocking agent would need to be shorter acting.

References

COLLIER, H.O.J., PARIS, S.K. & WOOLF, L.I. (1948). Pharmacological activities in different rodent species of d-tubocurarine chloride and the dimethyl ether of d-tubocuraine iodide. *Nature, Lond.*, 161, 817-819.

HUGHES, R. & CHAPPLE, D.J. (1975) Effects of non-depolarizing neuromuscular blocking agents on vagal and sympathetic mechanisms in cats. *Br. J. Anaesth.*, 47, 633-634.

MOGEY, G.A. & TREVAN, J.W (1950). D-tubocurarine salts and derivatives. *Br med. J.*, 2, 216.

SUGAI, N., HUGHES, R. & PAYNE, J.P. (1975) The effect of suxamethonium and its interaction with gallamine on the indirectly elicited tetanic and single twitch contractions of skeletal muscle in man during anaesthesia. *Br. J. clin. Pharmac.*, 2, 391-402.

The enterohepatic circulation of ^{3}H-phenolphthalein in the rat

P.C. HIROM, P. MILLBURN & R.J.PARKER*
(introduced by R.T. WILLIAMS)

Department of Biochemistry, St. Mary's Hospital Medical School, London W2 1PG

Many drugs are excreted in bile and, consequently, may undergo an enterohepatic circulation (EHC) (see Smith & Millburn, 1975). In the rat, phenolphthalein is conjugated with glucuronic acid and then extensively excreted in bile (Millburn, Smith & Williams, 1967). This paper reports on the EHC of phenolphthalein.

In bile-duct-cannulated rats injected with [^{3}H]-phenolphthalein (25 mg/kg i.p.), 89 ± 1.6% ($n = 3$) of the ^{3}H was excreted in bile in 3 h, whereas in intact rats four days are required for the elimination of 86 ± 3.9% ($n = 3$) in faeces. This delayed faecal excretion appears to be due to EHC.

Following intraduodenal infusion of [^{3}H]-phenolphthalein into bile-duct-cannulated rats, there is a rapid biliary excretion of radioactivity (Figure 1a) By contrast, on infusion of bile containing [^{3}H]-phenolphthalein glucuronide obtained from rats injected with [^{3}H]-phenolphthalein, there is a lag period of some 4 h before a comparable rate of excretion occurs (Figure 1a). This suggests that the glucuronide may require hydrolysis to the aglycone before significant absorption occurs, as is the case for stiboestrol glucuronide (Fischer, Millburn, Smith & Williams, 1966).

Rats were treated daily for 3 days with antibiotics to suppress the intestinal microflora. Figure 1b shows that this treatment did not inhibit the absorption of free phenolphthalein. However, on the intraduodenal infusion of bile containing [^{3}H]-phenolphthalein glucuronide the biliary excretion of ^{3}H (Figure 1b) was much lower than in untreated animals (Figure 1a). This indicates that hydrolysis of phenolphthalein glucuronide by bacterial β-glucuronidase is an important step in the EHC of phenolphthalein.

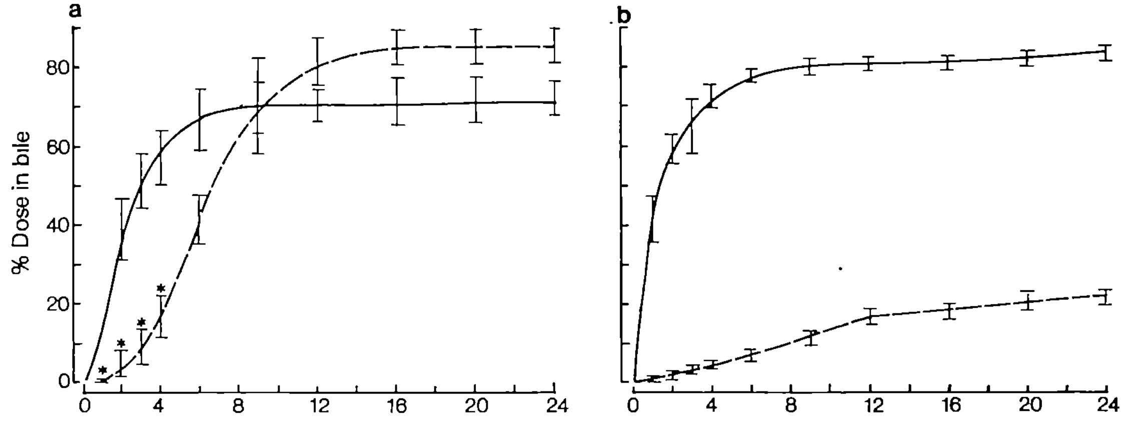


Figure 1 Biliary excretion of radioactivity after intraduodenal infusion of ^{3}H-phenolphthalein (——) or ^{3}H-phenolphthalein glucuronide (— — —) into bile-duct-cannulated female Wistar albino rats (200-250 g body weight). The dose was 79 µmol/kg. The vertical bars represent s.e. mean ($n = 3$).

a without antibiotic treatment. *These values are significantly ($P < 0.05$) lower than the corresponding values for phenolphthalein

b after 3 days treatment with neomycin (100 mg kg^{-1} day^{-1}), tetracycline (50 mg kg^{-1} day^{-1}) and bacitracin (50 mg kg^{-1} day^{-1}).

Carotid arterial blood level measurements suggest that phenolphthalein may be systemically bioavailable from the EHC. Thus, in intact but not in bile-duct-cannulated rats there is a secondary plasma peak of radioactivity 5-6 h after the intravenous administration of [^{3}H]-phenolphthalein. This peak coincides with the absorption of the aglycone from the intestine following the bacterial hydrolysis of phenolphthalein glucuronide (see Figure 1a).

R.J P. is grateful to the Medical Research Council for a research studentship. We are grateful to Professor R.T. Williams for his interest in this work.

References

FISCHER, L.J., MILLBURN, P., SMITH, R L & WILLIAMS, R T. (1966) The fate of ^{14}C-stilboestrol in the rat. *Biochem. J*, **100**, 69P.

MILLBURN, P., SMITH, R.L. & WILLIAMS, R T. (1967). Biliary excretion of foreign compounds Biphenyl, stilboestrol and phenolphthalein in the rat molecular weight, polarity and metabolism as factors in biliary excretion. *Biochem. J.*, **105**, 1275-1281.

SMITH, R L. & MILLBURN, P. (1975). Enterohepatic circulation and drug bioavailability. In *Proceedings of the Sixth International Congress of Pharmacology, Helsinki, Finland* (In press).

The effects of cyproheptadine pretreatment on insulin release from isolated pancreatic islets

B.P. RICHARDSON (introduced by B BERDE)

Biological and Medical Research Division, Sandoz Ltd., Basle Switzerland

Cyproheptadine, an antiserotonin-antihistaminic agent with a chemical structure similar to the tricyclic antidepressants (Stone, Wenger, Ludden, Stavorski & Ross, 1961) inhibits glucose-mediated insulin release by an immediate and direct effect on the rat pancreatic islet of Langerhans (Richardson, McDaniel & Lacy, 1975).

The present studies describe the effects of different secretogogues on insulin release from cyproheptadine-pretreated rat islets. Approximately 200 islets were isolated from two 200-300 g ten week old male albino rats (OFA Sandoz SPF strain) by the collagenase technique (Lacy & Kostianovsky, 1967). An equal number of islets were placed in each of two perfusion chambers and perifused at 37° C and pH 7.40 with Krebs Ringer bicarbonate containing 5.6 mM D-glucose at a rate of 1 ml/min as described previously (Lacy, Walker & Fink, 1972). After 45 min, the test islets were exposed to 100 μM cyproheptadine hydrochloride monohydrate for five minutes Subsequently both chambers were stimulated with an insulin secretogogue for a further 60 min as indicated in the Table The perfusate was collected at 1- or 5-min intervals throughout the study. The insulin content was determined by radioimmunoassay (Wright, Makulu, Vichick & Sussman, 1971) and expressed as (μU/islet)/minute. All data was subjected to complete statistical analysis.

Cyproheptadine pretreatment completely abolished tolbutamide- or glucose-evoked insulin release. Conversely the responsiveness of islets to

Table 1 The effects of cyproheptadine pretreatment on insulin release from perifused islets

Mean rate of secretion with 5.6 mM D-glucose (μU/islet)/min ± s.e.		*Insulin secretogogue added*	*n*	*Mean rate of secretion after addition of secretogogue μu/islet per min ± s.e. mean*		**P*	*% Inhibition*
Control	*Test*			*Control*	*Test*		
0.59 ± 0.04	0.54 ± 0.09	1.1 mM tolbutamide	3	1.37 ± 0.08	0.48 ± 0.09	< 0.001	100.0
0.47 ± 0.21	0.78 ± 0.29	11.1 mM D-glucose	3	2.67 ± 0.65	0.72 ± 0.22	< 0.01	100.0
0.73 ± 0.15	0.67 ± 0.08	11.1 mM D-glucose + 6.0 mEq/1. Ca^{++}	3	3.17 ± 0.37	0.96 ± 0.15	< 0.001	88.5
0.53 ± 0.35	0.49 ± 0.24	5.0 mM theophylline	4	1.19 ± 0.44	1.17 ± 0.33	NS	0.0

* Control versus test values after addition of secretogogue
NS = not significant

theophylline remained unaltered Theophylline elevates endogenous cyclic AMP in pancreatic β-cells, thus producing liberation of calcium from intracellular bound pools, with resultant insulin release (Brisson, Malaisse-Lagae & Malaisse, 1972). It seems unlikely, therefore, that cyproheptadine interferes with either cyclic AMP generation or with intracellular calcium metabolism. Although increasing the extracellular calcium concentration by an additional 6.0 mEq/l did not itself stimulate insulin release, it partially reversed the inhibition of glucose mediated release caused by cyproheptadine. Taken together, these results suggest that cyproheptadine inhibits the uptake of calcium by the β-cell without affecting its intracellular metabolism.

I am grateful to Dr M.E. Kitler for the statistical analysis of the data.

References

BRISSON, G.R., MALAISSE-LAGAE, F. & MALAISSE, W.J. (1972). The stimulus-secretion coupling of glucose-induced insulin release VII. A proposed site of action for adenosine 3',5'-cyclic monophosphate. *J. clin. Invest*, **51**, 232-241.

LACY, P E & KOSTIANOVSKY, M. (1967). Method for the isolation of intact islets of Langerhans from the rat pancreas. *Diabetes*, **16**, 35-39.

LACY, P.E., WALKER, M M. & FINK, J. (1972). Perfusion of isolated rat islets *in vitro* *Diabetes*, **21**, 987-988.

RICHARDSON, B.P, McDANIEL, M & LACY, P.E. (1975). Effects of cyproheptadine on insulin secretion by isolated perifused rat islets. *Diabetes* (in press).

STONE, C.A., WENGER, H.C., LUDDEN, C.T., STAVORSKI, J.M. & ROSS, C.A (1961). Antiserotonin-antihistaminic properties of Cyproheptadine. *J. Pharmac. exp. Ther.*, **131**, 73-84.

WRIGHT, P.H., MAKULU, D.R., VICHICK, D. & SUSSMAN, K.E. (1971). Insulin immunoassay by back titration, some characteristics of the technic and the insulin precipitant action of alcohol. *Diabetes*, **20**, 33-45.

Effects of dibutyryl cyclic AMP and phosphodiesterase inhibitors on acid secretion by mouse stomach *in vitro*

BEATRICE Y.C. WAN (introduced by J.W. BLACK)

Department of Pharmacology, University College London, Gower Street, London WC1E 6BT

Kimberg (1974) has reviewed the evidence about the involvement of cyclic AMP in histamine- or pentagastrin-stimulated gastric acid secretion *in vivo* in the rat. In the present investigation quantitative studies on the effects of dibutyryl cyclic AMP on gastric acid secretion have been carried out *in vitro* in the presence and absence of metiamide, a specific histamine H_2-receptor antagonist. The effects of a potent phosphodiesterase inhibitor, a triazolopyrimidine 2-amino-6-methyl-5-oxo-4-*n*-propyl-4,5-dihydro-*s*-triazolo(1,5-a) pyrimidine (ICI 63197) on the acid secretory responses to histamine and pentagastrin have also been examined.

As the stomach wall of immature mouse is very thin, an isolated whole mouse stomach was considered suitable for *in vitro* studies. Mice (Charles River) of either sex, 2-6 weeks old, were anaesthetized with ether. The stomach was washed with warm saline by way of incisions made at the pyloric and cardiac regions. A glass bead was introduced into the lumen to reduce dead space. The oesophagus was ligated and polythene cannulae were tied into the pyloric sphincter and the cardiac region. The isolated stomach was placed in an organ bath at 37° C containing a buffered solution (Davenport, 1951) gassed vigorously with 95% O_2 + 5% CO_2. The stomach lumen was perfused at 1 ml/min with unbuffered solution gassed with 100% O_2. The lumen perfusate was passed over a flow-type glass micro-electrode and pH changes were continuously recorded. The results were expressed as peak acid secretion $[H^+]$ μM.

Basal secretion usually reached a steady level after incubation for forty minutes. Dibutyryl cyclic AMP(db cyclic AMP) regularly stimulated acid secretion in a dose-dependent manner. Since tachyphylaxis to repeated exposure to db cyclic AMP was sometimes observed, only results for acid secretory response to the first dose of db cyclic AMP have been used for statistical analysis The results show that in the presence of db cyclic AMP 10^{-4}, 2.5 x 10^{-4}, 5 x 10^{-4}, and 10^{-4} M, hydrogen ion concentrations rose from a mean ± s.e. mean basal level of 35.3 ± 4.2 ($n = 19$) μM to 73.9 ± 10.9(3), 87.8 ± 9.5(7), 114.8 ± 10.4(3) and 299 ± 29 1(6) respectively.

Paired 't' tests on results from four experiments showed that within preparations, there was no significant difference between the acid secretory responses to db cyclic AMP (2.5×10^{-4} M) in the absence and presence of metiamide (up to 5×10^{-4} M). Student's 't' test on results from different preparations also indicated that metiamide did not inhibit acid secretion induced by db cyclic AMP ($n = 5$ for db cyclic AMP, 2.5×10^{-4} M, and for metiamide up to 5×10^{-4} M + db cyclic AMP 2.5×10^{-4} M). In contrast to db cyclic AMP, histamine or pentagastrin did not regularly stimulate acid secretion. However, marked stimulation of acid secretion by histamine or pentagastrin could be obtained in the presence of phosphodiesterase inhibitors. The order of effectiveness was triazolopyrimidine > theophylline > caffeine. In preparations treated with histamine 20 min after triazolopyrimidine (10^{-4} M) was added, sustained and dose-related responses to histamine (10^{-5} to 10^{-3} M) could be obtained. In the presence of triazolopyrimidine (10^{-4} M), [H^+] secretion to a submaximal dose of pentagastrin (2×10^{-6} M) was about 80 μM as compared to that of about 100 μM in response to a submaximal dose of histamine (2.5×10^{-4} M). Triazolopyrimidine on its own caused slight stimulation of acid secretion by the isolated mouse stomach.

The present results are in agreement with the findings of Fromm, Schwartz & Quijano (1975), who showed that marked stimulation of acid secretion in the isolated rabbit gastric mucosa by db cyclic AMP was not inhibited by metiamide. The exact mode of action of phosphodiesterase inhibitors on the acid secretory responses to histamine and pentagastrin is not clear. In view of the evidence that triazolopyrimidine greatly potentiated the increase in cyclic AMP levels induced by biogenic amines in mouse cerebral slices (Nahorski & Rogers, 1975), the present results support the hypothesis that cyclic AMP may be involved in histamine or pentagastrin-induced acid secretion by the mouse stomach *in vitro*.

References

DAVENPORT, H W. (1951). Methods for in vitro study of gastric acid secretion. In *Methods in Medical Research* Vol 4. pp. 184-190

FROMM, D , SCHWARTZ, J.H. & QUIJANO, R. (1975) Effects of cyclic adenosine 3' · 5'-monophosphate and related agents on acid secretion by isolated rabbit gastric mucosa *Gastroenterology*, 69, 453-462

KIMBERG, D V. (1974). Cyclic nucleotides and their role in gastrointestinal secretion. *Gastroenterology*, 67, 1023-1064.

NAHORSKI, S.R. & ROGERS, K.J (1975). The effect of phosphodiesterase inhibitors on the stimulation of cerebral cyclic AMP formation by biogenic amines in vitro and in vivo. *Br. J. Pharmac.*, 54, 272P

The effects of intravenous secretin on the small intestinal vasculature of the cat

P.D.I. RICHARDSON

Department of Physiology, The Medical College of St. Bartholomew's Hospital, Charterhouse Square, London, EC1M 6BQ

Secretin, administered intravenously or intra-arterially, increases superior mesenteric arterial blood flow in the anaesthetized cat (Ross, 1970, Fasth, Filipsson, Hulten & Martinson, 1972). Since other gastrointestinal hormones such as glucagon and pentagastrin, when infused intravenously in low doses, alter capillary filtration coefficient in the cat small intestine (Richardson, 1975), the effects of intravenous secretin on the resistance, capacitance, and vascular exchange function of this tissue have been examined.

Eight cats (2.66-3.87 kg) were anaesthetized with α-chloralose (70 mg/kg i.v.) after halothane induction, and loops of sympathetically-innervated jejunum (62.6 ±18.4 g, mean ± s.d) prepared for the measurement of blood flow, changes in tissue volume, and capillary filtration coefficient (CFC), by a modification (Richardson, 1974) of the plethysmographic technique of Folkow, Lundgren & Wallentin (1963).

Under control conditions, the systemic arterial mean pressure (BP) was 151.4 ± 4.2 (mean ± s.e. mean) mmHg, the heart rate (HR) 174.6 ± 10.0 beats/min, and the superior mesenteric venous outflow (SMVF) 55.7 ± 7.8 ml min^{-1} 100 g^{-1}; giving a calculated jejunal vascular resistance (JVR) of 3.15 ± 0.57 mmHg ml^{-1} min 100 g. The CFC, measured as the continuous volume increase resulting from raising the superior mesenteric venous pressure by 10 cm H_2O for 1 min was 0.060 ± 0.009 ml min^{-1} mmHg^{-1} 100 g^{-1} ($n = 5$).

Natural secretin (Boots) was infused intravenously in a dose of 0.1 U kg^{-1} min^{-1} (Crick-Harper-Raper Units; 1 U = 62.5 ng) to five preparations, resulting in a significant (paired 't'-test, $P < 0.05$) increase in CFC of 38.8 ± 9.2% from 0.060 ± 0.009 to 0.083 ± 0.012 ml min^{-1} mmHg^{-1} 100 g^{-1}. The jejunal volume (JV) rose by 0.27 ± 0.06 ml/100 g at the start of the infusion

($P < 0.05$) but changes in BP, HR, SMVF and JVR were variable and insignificant ($P > 0.30$).

When secretin was infused intravenously in a dose of 0.5 U kg^{-1} min^{-1} to five preparations, the CFC rose by 61.6 ± 10.9% from 0.060 ± 0 009 to 0 095 ± 0.011 ml min^{-1} $mmHg^{-1}$ 100 g^{-1} ($P < 0.02$), and the JV rose by 0.83 ± 0.25 ml/100 g ($P < 0.05$). These infusions resulted in falls in BP (3.4 ± 1.2%), rises in HR (13.5 ± 5.0%), rises in SMVF (17.4 ± 7.2%) and reductions in JVR (16.4 ± 5.5%), changes which were consistent, but not statistically significant ($P > 0.05$). The effects of secretin at this dose on CFC and JV were not modified by pretreatment with hexamethonium (5 mg/kg, i.v.: three experiments) or propranolol (0.1 mg/kg, i.v.: three experiments).

On six occasions in three preparations, intravenous injections of 0.02 to 20.0 U/kg secretin resulted in dose-dependent transient reductions in JVR (maximum. −67.3 ± 3.0%) and increases in JV (maximum: +1.61 ± 0.27 ml/100 g). Doses above 0.5 U/kg additionally caused dose-dependent falls in BP and rises in HR.

As well as reducing small intestinal vascular resistance, intravenous secretin causes an increase in tissue volume over the same dose range, indicating dilatation of capacitance vessels (Folkow *et al.*, 1963). Low dose infusions increase CFC and JV without significant effects on other variables: the rise in CFC indicates either dilatation of precapillary 'sphincters', leading to an increased functional exchange vessel area, or an increased vascular permeability, mechanisms which are not separable by this technique.

This study was generously supported by the Medical Research Council; the skilful technical assistance of Miss Dorinda Lobendhan and Miss June Pyke is gratefully acknowledged.

References

FASTH, S., FILIPSSON, S, HULTEN, L. & MARTINSON, J. (1972). The effect of gastrointestinal hormones on small intestinal motility and blood flow. *Experientia*, 29, 982-984.

FOLKOW, B., LUNDGREN, O. & WALLENTIN, I. (1963) Studies on the relationship between flow resistance, capillary filtration coefficient and regional blood volume in the intestine of the cat. *Acta physiol. scand.*, 57, 270-283.

RICHARDSON, P.D.I. (1974). Drug-induced changes in capillary filtration coefficient and blood flow in the innervated small intestine of the anaesthetized cat. *Br. J. Pharmac.*, 52, 481-498.

RICHARDSON, P.D.I. (1975). The effects of glucagon and pentagastrin on capillary filtration coefficient in the innervated jejunum of the anaesthetized cat. *Br. J. Pharmac.*, 54, 225P.

ROSS, G. (1970). Cardiovascular effects of secretin. *Am. J. Physiol.*, 218, 1166-1170.

Some preservatives in eyedrop preparations hasten the formation of dryspots in the rabbit cornea

A.J. DUNCAN & W.S. WILSON*

Department of Pharmacology, Glasgow University, Glasgow G12 8QQ

Three factors are important in the maintenance of the tear film between the replenishing action of the blink. Its adherence to the cornea is aided (a) by the surface microplicae on the epithelial cells, (b) by the surface-tension lowering layer of conjunctival mucus which overlies the epithelium and (c) by an outer layer of oil (secreted by the Meibomian glands) which delays evaporation. Since surface-active substances are often used as preservatives in eyedrop preparations, these experiments were undertaken to examine the effect of such substances on the stability of the rabbit tear film.

Dutch rabbits were anaesthetized with halothane (3%) in nitrous oxide-oxygen (3 : 1). Saline (3 drops of 0.9% w/v) was applied to both eyes at zero time and the excess fluid removed. The time taken for the development of a dry spot on each cornea was measured and was taken as the 'start-control' value (each eye to act as its own control). The tear film was immediately restored by blinking the eyelids three times. Three drops of preservative solution (lowest concentration, in 0.9% saline or suitable vehicle) were then applied, excess fluid removed and the drying time measured again. This procedure was repeated for successive increases in concentration of the preservative solution. Finally, the cornea was irrigated with saline and the eyelids repeatedly blinked for 10 min, before another control determination was made ('end-control').

The start-control value (mean ± s.e. mean) was 151.4 ± 20.1 s ($n = 81$). Variation in control times from animal to animal greatly exceeded that between L and R eyes of each rabbit, while variation between start-control and end-control times was not statistically significant. Several preservatives, in concentrations equivalent to those used in eyedrop preparations, produced statistically significant hastening of the drying time. 0.01% (w/v) benzalkonium chloride (22.9% of start-control time); 0.03% *n*-propyl *p*-hydroxybenzoate (54 1%); 0.3% 2-phenylethanol (53.1%); 0.01% chlorhexidine (38.2). All of these substances produced dose-related decreases in drying time at the lower concentrations tested, in some cases down to one-hundredth the concentration indicated. Two mercurial preservatives, 0.3% (w/v) thiomersal and 0.1% phenylmercuric nitrate, caused no change in the rate of corneal drying.

A time-course study indicated that the effect of applying 2 drops of 0.01% benzalkonium chloride could still be detected 45 min afterwards. The presence of 0.01% benzalkonium chloride also significantly hastened the drying time following application of 0.3% or 1.5% (w/v) hydroxypropylmethylcellulose drops.

An approximate relationship was shown to exist between the ability of these substances to lower surface tension and to hasten the formation of dryspots on the cornea. This indicates that the mechanism of this effect may involve adsorption to, or solubilization of, either the conjunctival mucus layer or the oily Meibomian secretion.

The effect of melatonin on pinealectomy-induced hypertension in the rat

S.W. HOLMES & D. SUGDEN*

Department of Pharmacology, Roche Products Ltd., Welwyn Garden City, Hertfordshire

Surgical removal of the pineal gland has been shown to elevate blood pressure in anaesthetized rats (Zanoboni & Zanoboni-Muciaccia, 1967) and a modest hypertension (20 mmHg) has been described in unanaesthetized rats after electrocoagulation of the pineal gland (Karppanen, Vapaatalo, Lahovarra & Paasonen, 1970). These authors suggest that an increased adrenal steroid level after pinealectomy contributes to the observed hypertension. Karppanen, Airaksinen & Särkimäki (1973) have proposed that melatonin and/or related pineal hormones may act as natural anti-hypertensive agents, possibly by stimulating central inhibitory adrenergic pathways

In the present study the effect of melatonin administration on hypertension produced by electrolytic lesion of the pineal gland in the rat was investigated.

Male Sprague-Dawley rats (180-220 g), anaesthetized with pentobarbitone sodium (60 mg/kg i.p.), had a 21 g hypodermic needle positioned 2.0 mm vertically below the surface of the skull at lambda. A d.c. current of 40 mA passed between the needle and a negative electrode on the ear for 20 s was found to produce destruction of the pineal gland with minimal damage to surrounding cortical tissue. Destruction of the pineal gland was verified macroscopically in all animals at the end of the experiment. Sham-operated rats had identical electrode placement but no current was applied. Systolic blood pressure was measured indirectly from unanaesthetized rats using the tail-cuff method.

A modest, but statistically significant, hypertension (15-20 mmHg) was seen in the pinealectomized animals ($n = 16$), compared to sham-operated or unoperated controls, from 1 week after operation until at least 7 weeks. Melatonin administered in the drinking water [1 mg/ml in 1.25% ethanol vehicle started immediately after operation] prevented the emergence of pinealectomy-induced hypertension and significantly depressed the blood pressure to below that of vehicle-treated control animals. Replacement of drug treatment with vehicle alone resulted in the development of hypertension in these pinealectomized animals. Administration of melatonin to animals in which hypertension had become established resulted in a significant fall of blood pressure to below sham-operated levels after 2 weeks of treatment. It was estimated that the average daily intake of melatonin for each rat was approximately 100 mg/kg.

These results suggest that the hypertension caused by pinealectomy is, at least in part, due to the lack of normal melatonin secretion by the gland.

References

KARPPANEN, H., VAPAATALO, H., LAHOVARRA, S. & PAASONEN, M.K. (1970). Studies with pinealectomized rats. *Pharmacology*, 3, 76-84.

KARPPANEN, H., AIRAKSININ, M.M. & SÄRKIMÄKI, I. (1973). Effects in rats of pinealectomy and oxypertine on spontaneous locomotor activity and blood pressure during various light schedules. *Ann. Med. exp. Biol. Fenn*, 51, 93-103,

ZANOBONI, A. & ZANOBONI-MUCIACCIA, W (1967) Experimental hypertension in pinealectomized rats. *Life Sci.*, 6, 2327-2331.

BRL 13776: a novel antihypertensive agent with interesting noradrenaline-depleting properties

JOANNE MELROSE, M.G. PALFREYMAN*
R.H POYSER & R.L. WHITING

Beecham Pharmaceuticals, Research Division, Medicinal Research Centre, Coldharbour Road, The Pinnacles, Harlow, Essex, CM19 5AD

Whilst studying a series of 4-(4-pyridyl)-chroman-5-ols which were being evaluated for activity in the central nervous system, a compound BRL 13776 (7-n-pentyl-4-[1-(2-naphthylmethyl)-1, 2, 5, 6-tetrahydro-4-pyridyl]-2, 2-dimethylchroman-5-ol) was found to have antihypertensive activity yet be devoid of behavioural properties The antihypertensive activity was assessed in deoxycorticosterone acetate/NaCl-treated hypertensive rats (Sprague-Dawley) and renal hypertensive cats (cellophane perinephritis model, see Poyser, Shorter & Whiting, 1974). In both models, BRL 13776 (suspended in 1% w/v methylcellulose) caused a significant lowering ($P < 0.05$) of blood pressure at doses of 30 mg/kg p.o. and above. The antihypertensive response was evident 4-6 h after dosing and blood pressure had almost returned to pre-dose values at 24 hours. No behavioural changes nor adverse symptoms were observed in either species.

Studies on the mechanism of action of BRL 13776 revealed that the compound depleted noradrenaline in various peripheral organs of both normotensive and hypertensive rats. Noradrenaline was measured by the method of Shellenberger & Gordon (1971), and animals receiving BRL 13776 were compared with vehicle-dosed controls. Six or more rats were used in each group and all animals were killed 6 h after dosing. In the normotensive rats, a single dose of BRL 13776 (100 mg/kg p.o.) reduced the noradrenaline content of heart, spleen and adrenals by 78%, 80% and 82% respectively ($P < 0.001$). A similar depletion occurred in hypertensive rats (e.g 65% in the heart, $P < 0.001$).

In contrast to the depletion in peripheral tissues, BRL 13776 did not affect the noradrenaline content of whole brain Nevertheless, more detailed studies revealed a significant reduction ($P < 0.01$) in the hind-brain region (pons/medulla) of both normotensive and hypertensive rats. This was in the order of 37-40% at 6 h following the 100 mg/kg single p.o. dose No concomitant decrease in the noradrenaline concentration of the cerebral hemispheres, mid-brain or hypothalamus was observed. In this respect BRL 13776 differed from reserpine. Even on repeated dosage with BRL 13776 (100 mg/kg p.o. twice daily for 14 days) the cerebral hemispheres were not depleted of their noradrenaline whereas the reduction in the hind-brain was still apparent (18%, $P < 0.01$). However, in the repeated-dose study there was also some depletion of the hypothalamus (28%, $P < 0.01$). Throughout the 14 day study no gross toxicity, behavioural changes or side effects were observed.

In conclusion, BRL 13776 is a novel structure displaying antihypertensive and noradrenaline-depleting properties. Noradrenaline depletion in the brain of rats is restricted to certain areas, and this may be relevant to both the antihypertensive response and the lack of behavioural effects. Reductions of noradrenaline in the periphery could contribute to the antihypertensive response or on the other hand be totally responsible. Obviously BRL 13776 warrants further investigation and it is intended to proceed to clinical studies.

References

POYSER, R H., SHORTER, J.H. & WHITING, R.L. (1974). The production of hypertension and the effects of some antihypertensive agents in the conscious cat. *Br. J. Pharmac.*, 51, 149P.

SHELLENBERGER, M.K. & GORDON, J H. (1971) A rapid, simplified procedure for simultaneous assay of norepinephrine, dopamine and 5-hydroxytryptamine from discrete brain areas. *Anal. Biochem.*, 39, 356-372.

Two types of intrinsic sympathomimetic activity with β-adrenoceptor blocking drugs.

A. BILSKI* & J. WALE (introduced by P.M. DAWES)

ICI (Pharmaceuticals) Ltd., Alderley Park, Macclesfield, Cheshire

The intrinsic sympathomimetic activity of β-adrenoceptor blocking drugs can be demonstrated by an increase in heart rate in rats depleted of catecholamines by pretreatment with syrosingopine. Thus dichlorisoprenaline, practolol, oxprenolol and pindolol produce a dose dependent tachycardia. The relationship between the dose requirements for intrinsic sympathomimetic activity and β-adrenoceptor blockade is not however uniform. Dichlorisoprenaline and practolol demonstrated intrinsic activity at all β-adrenoceptor blocking doses whereas the intrinsic activity of both oxprenolol and pindolol was evident only at high doses, relative to β-adrenoceptor blockade.

The haemodynamic effects of practolol and pindolol were compared in anaesthetized dogs with those of propranolol and 4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetamide (ICI 66082), which are without intrinsic sympathomimetic activity. The depression of heart rate and atrio-ventricular conduction time, aortic flow and left ventricular dp/dt (during cardiac pacing) produced by practolol, was less than that resulting from either propranolol or ICI 66082. Pindolol however produced a biphasic response with depression of cardiac function of a similar order to that produced by propranolol at a dose of 0.025 mg/kg but a tendency to increase cardiac function at a dose of 1 mg/kg. These results therefore also support the conclusion from the work in rats that the intrinsic sympathomimetic activity of pindolol is only apparent at high doses relative to β-adrenoceptor blockade. The intrinsic activity of practolol is present at all β-adrenoceptor blocking doses and effectively reduces the haemodynamic depressant effects of β-adrenoceptor blockade.

An interaction between the α and β actions of catecholamines in guinea-pig liver slices

K. KOLLER (introduced by D.H. JENKINSON)

Department of Pharmacology, University College London, Gower Street, London WC1E 6BT

Catecholamines have been shown to increase glucose release and potassium efflux from guinea-pig liver slices (Haylett & Jenkinson, 1972). The membrane potential of cells within the slice also rises, and this, together with the change in potassium efflux, can be accounted for as a consequence of an increase in the potassium permeability of the liver cells. The effect on potassium permeability is normally mediated by an α-adrenoceptor-like receptor. However, low doses of isoprenaline (50 nM), which generally have little effect on the membrane potential, have been found to cause hyperpolarization if applied up to 20 min after application of the selective α-adrenoceptor agonist amidephrine (Haylett & Jenkinson, 1973). For convenience this phenomenon has been termed potentiation.

The aim of the present work was to examine the pharmacological characteristics of the potentiated response, and the results to be described are based on comparisons of the effects of drugs on the continuously recorded membrane potentials of individual cells in guinea-pig liver slices (for methods see Haylett & Jenkinson, 1972).

The main series of experiments was designed to test whether potentiation could be observed with other α- and β-adrenoceptor agonists. Cyclic AMP and its dibutyryl derivative were also examined, in view of the possible role of cyclic AMP in responses mediated by β-adrenoceptors.

The following agents were found to cause hyperpolarization and potentiation in the dose range 5-50 μM, and both effects could be reversibly abolished by phentolamine (10 μM), suggesting that these actions are α-mediated and related: amidephrine, methoxamine, naphazoline, noradrenaline, oxymetazoline, phenylephrine, tetrahydrozoline, tramazoline, xylometazoline.

The additional finding that α-adrenoceptor agonists potentiated the responses not only to isoprenaline and salbutamol but also to cyclic AMP (Figure 1a and 1b) and dibutyryl cyclic AMP suggests that potentiation of β-mediated responses occurs at a step subsequent to β-adrenoceptor activation. In keeping with this idea, 10 μM propranolol reversibly abolished the potentiated response to isoprenaline and salbutamol but had no effect on that to cyclic AMP.

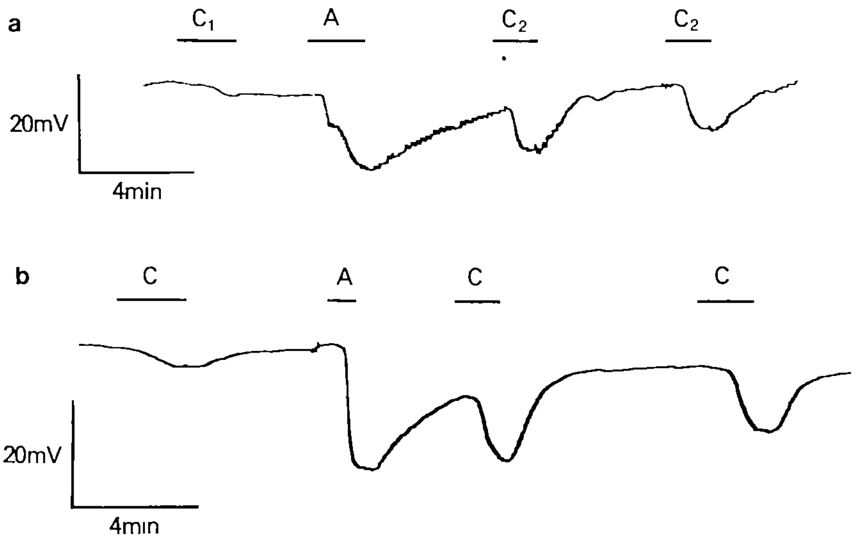


Figure 1 Figure *1a* shows responses to cyclic AMP (1 mM at 'C_1' and 0 25 mM at 'C_2') before and after an application of the α-agonist (−)amidephrine (10 μM at 'A') Figure *1b* is from another experiment where the chloride in the bathing fluid had been replaced by isethionate, and shows that potentiation of cyclic AMP (0.5 mM at 'C') by (−)amidephrine (10 μM at 'A') is not chloride-dependent

I wish to thank the Medical Research Council for financial support.

References

HAYLETT, D G. & JENKINSON, D.H. (1972). Effects of noradrenaline on potassium efflux, membrane potential and electrolyte levels in tissue slices prepared from guinea-pig liver. *J. Physiol, Lond*, 225, 721-750.

HAYLETT, D.G. & JENKINSON, D.H (1973). Actions of catecholamines on the membrane properties of liver cells. In *Drug Receptors*, ed. Rang, H P., pp 15-25. London: The Macmillan Press Ltd.

Alterations to vasodilator effects of isoprenaline in the dog following intra-arterial infusions with isoprenaline, salbutamol and orciprenaline

P.S. COLLIER* & R.G. SHANKS

Department of Pharmacy and Department of Therapeutics and Pharmacology, The Queen's University of Belfast, Northern Ireland

Recently there has been much interest in the possible development of tolerance to sympathomimetic bronchodilator drugs (Conolly, Davies, Dollery & George, 1971; Minatoya & Spilker, 1975).

In the present studies the effects of infusions of three sympathomimetic drugs on the blood flow response to isoprenaline have been investigated in twenty anaesthetized dogs.

Heart rate, arterial blood pressure and blood flow in the external iliac artery were recorded. In each dog, isoprenaline (0 001, 0.002, 0.004 and 0.008 μg/kg) was injected into the external iliac artery and the increase in blood flow noted. The maximum dose of isoprenaline injected was such that changes in heart rate and arterial blood pressure were minimal.

In group 1, four successive dose response curves were constructed allowing 40 min between each. The remaining three groups received an infusion into the external iliac artery for 30 min between successive dose response curves. Group 2 was infused with isoprenaline 0.002 μg kg^{-1} min^{-1} between dose response curves 1 and 2, 0.006 μg kg^{-1} min^{-1} between dose response curves

2 and 3 and 0.018 $\mu g\ kg^{-1}\ min^{-1}$ between dose response curves 3 and 4. Groups 3 and 4 were infused in a similar manner to group 2 with salbutamol 0.002, 0.006 and 0.018 $\mu g\ kg^{-1}\ min^{-1}$ and orciprenaline 0 02, 0.06 and 0 18 $\mu g\ kg^{-1}\ min^{-1}$ respectively.

A decrease in the blood flow response was seen with similar doses of isoprenaline in successive dose response curves of each group This was significant only in group 2 ($P < 0\ 01$) and group 3 ($P < 0\ 001$) Isoprenaline and orciprenaline infusions at the dose levels used produced equivalent increases in blood flow but the decrease in responsiveness to isoprenaline was not as great in group 4 as in group 2. Salbutamol infusions produced the smallest increase in blood flow but caused the greatest decrease in blood flow response to isoprenaline.

Thus the decrease in responsiveness to isoprenaline that followed the infusions with isoprenaline and salbutamol did not relate to the increase in flow caused by the infusions, nor could it be explained in terms of duration of action, as all infusions were for 30 min and the resting flow rate was regained within 10 min following the infusions

References

CONOLLY, M.E., DAVIES, D.S., DOLLERY, C.T. & GEORGE, C.F. (1971) Resistance to β-adrenoceptor stimulants (a possible explanation for the rise in asthma deaths). *Br. J. Pharmac.*, 43, 389-402.

MINATOYA, H & SPILKER, B A (1975) Lack of cardiac or bronchodilator tachyphylaxis to isoprenaline in the dog. *Br J. Pharmac*, 53, 330-340

The effects of AH 5158 on the overflow of transmitter and the uptake of $[^3H]$-(—)-noradrenaline in the cat spleen

A.G.H. BLAKELEY & R.J. SUMMERS*

Department of Pharmacology, The University, Glasgow G12 8QQ

AH 5158, an adrenoreceptor blocking drug, produces competitive blockade of both α and β adrenoceptors (Farmer, Kennedy, Levy & Marshall, 1972, Kennedy & Levy, 1975) The effects of this drug on the overflow of transmitter following nerve stimulation with 200 stimuli at 10 and 30 Hz have been examined in the isolated blood perfused cat spleen At 30 Hz, concentrations up to 10^{-4} M of the drug produced a dose-dependent elevation of transmitter overflow and a potentiation of the vascular response of the spleen. Higher concentrations (1-4.2 x 10^{-4} M) decreased transmitter overflow and depressed the vascular response At 10 Hz, 5-(1-hydroxy-2-((1-methyl-3-phenylpropyl)amino)ethyl)salicylamide (AH 5158) (3.3 x 10^{-5} M) elevated transmitter overflow from 264 ± 56 pg/stim to 556 ± 109 pg/stim ($P < 0.05, n = 8$).

Two explanations for the elevation of overflow produced by the lower concentrations are inhibition of transmitter uptake and the α-receptor mediated feedback controlling transmitter liberation (Langer, 1974).

AH 5158 (1.5 x 10^{-4} M) inhibited noradrenaline uptake increasing the recovery of $[^3H]$ in the venous blood during close arterial infusion of $[^3H]$-(−)-noradrenaline (370 ng/min; blood flow 8 ml/min) from 51 ± 2% to 77 ± 4% ($P < 0.01$; $n = 4$). Uptake inhibition alone could account for the effects on overflow and response

In isolated strips of splenic capsule both AH 5158 (3.8 x 10^{-5} M) and the competitive α-adrenolytic 2-piperidinomethyl-1,4-benzodioxan hydrochloride (933F) (7.4 x 10^{-6} M) produced similar 10-fold parallel shifts to the right of the dose-response curve to (−)-noradrenaline. In the perfused spleen 933F (5 7 x 10^{-6} M) increased the overflow of transmitter following nerve stimulation at 10 Hz to 1506 ± 78 pg/stim ($n = 14$) by a mechanism involving only presynaptic α-adrenoceptor inhibition.

The local anaesthetic action of AH 5158 (Farmer *et al.*, 1972) could account for the depressant effect of high drug concentrations on transmitter overflow but is not important at 3.3 x 10^{-5} M since subsequent addition of 933F (8 x 10^{-6} M) increased the overflow at 10 Hz to 1306 ± 237 pg/stim ($n = 6$).

These experiments support the hypothesis that there are in the spleen presynaptic and post-synaptic α-adrenoceptors with different sensitivities to antagonist drugs. In the spleen AH 5158 is an inhibitor of noradrenaline uptake and an antagonist of postsynaptic α-adrenoceptors.

The authors thank the Rankin Fund for support.

References

FARMER, J B., KENNEDY, I, LEVY, G P. & MARSHALL, R.J. (1972). Pharmacology of AH 5158, a drug which blocks both α and β adrenoceptors. *Br J Pharmac.*, 45, 660-675.

KENNEDY, I. & LEVY, G.P. (1975). Combined α and β adrenoceptor blocking drug AH 5158 further studies on α-adrenoceptor blockade in anaesthetized animals. *Br. J. Pharmac.*, 53, 585-592

LANGER, S Z (1974). Presynaptic regulation of catecholamine release *Biochem. Pharmac*, 23, 1793-1800.

The sympathetic nervous system and renovascular hypertension in the rat

H.J. DARGIE*, S.S. FRANKLIN & J.L. REID

Department of Clinical Pharmacology, Royal Postgraduate Medical School, University of London

The integrity of central noradrenergic neurones is essential for the development and maintenance of DOCA salt hypertension in the rat (Haeusler, Finch & Thoenen, 1972, Dargie, Dollery & Lewis, 1975) and perinephritis hypertension in the rabbit (Chalmers, Dollery, Lewis & Reid, 1974). Plasma noradrenaline levels are increased in the DOCA salt model and the rise in both blood pressure and plasma noradrenaline can be prevented by pretreatment with intracisternal (i.c.) 6-hydroxydopamine (6-OHDA) (Dargie, Lewis, Reid & Zivin, 1975).

We have measured plasma noradrenaline (NA) as an index of peripheral sympathetic activity in a renovascular model of experimental hypertension in the rat and have assessed the relationship between central and peripheral sympathetic activity in the development of hypertension in this model.

Male Wistar rats (weighing 200 g) had a silver clip 0.007 in. wide placed over the left renal artery followed by contralateral nephrectomy. Control rats underwent a sham procedure involving placement of a broad non-constricting clip over the renal artery and contralateral nephrectomy. Blood pressure was measured by tail plethymography at 24 h, 7, 14 and 28 days respectively. The animals were killed by decapitation and the first 1 ml of arterial blood from the trunk was collected in iced heparin tubes for estimation of NA by the method of Henry, Starman, Johnson & Williams (1975).

Two groups of rats were pre-treated 14 days before with intracisternal 6-OHDA or ascorbate saline vehicle. Renal arterial clip or sham operations were carried out on each group. Blood pressure was measured after 7 days, the animals were decapitated and blood collected.

In the first series of experiments blood pressure was significantly elevated ($P < 0.01$) in the rats with renal arterial clips at 24 h, 7, 14 and 28 days. Mean systolic blood pressure (± s.e mean) ranged from 144 ± 5 mmHg at 24 h to a maximum of 177 ± 9 at 14 days. Blood pressures (in corresponding 'sham-operated' rats) were 109 ± 4 and 115 ± 4 mmHg. Plasma NA in the clip groups was higher than sham operated rats at each time examined. These differences were significant at 7, 14 and 28 days. At 14 days plasma NA was 1.91 ± 0.32 μg/ml in clip groups and 0.93 ± 0.24 ng/ml in sham operated controls ($P < 0.05$).

Blood pressure and plasma noradrenaline were elevated in the group of rats given i.c. vehicle and renal arterial clip. However, in the group pretreated with i.c. 6-OHDA blood pressure and plasma noradrenaline levels did not rise and were not significantly different from their controls.

It is concluded that peripheral sympathetic activity is increased in the one kidney Goldblatt model of experimental hypertension and that this increase in sympathetic activity is mediated by catecholamine mechanisms in the central nervous system.

References

CHALMERS, J.P., DOLLERY, C.T., LEWIS, P.J. & REID, J.L. (1974). The importance of central adrenergic neurones in renal hypertension in rabbits. *J. Physiol., Lond.*, 238, 403-411.

DARGIE, H.J., DOLLERY, C.T. & LEWIS, P.J. (1975). Role of saline consumption in the prevention of doca salt hypertension by central 6-hydroxydopamine; role of saline intake. *Br. J. Pharmac.*, 53, 455P.

DARGIE, H J., LEWIS, P.J., REID, J.L. & ZIVIN, J.A. (1975). Mechanism of the prevention of doca saline hypertension by central 6-hydroxydopamine. *Proceedings 6th International Congress of Pharmacology, Helsinki, Finland* (in press).

HAEUSLER, G., FINCH, L. & THOENEN, H. (1972). Central adrenergic neurones and the initiation and development of experimental hypertension. *Experientia*, 28, 1200-1203

HENRY, D.P., STARMAN, B J., JOHNSON, D.G. & WILLIAMS, K.N. (1975). A sensitive radioenzymatic assay for norepinephrine in tissues and plasma. *Life Sci.*, 16, 375-384.

Studies on the centrally mediated cardiovascular effects of apomorphine in the anaesthetized rat

L. FINCH & A. HERSOM*

School of Studies in Pharmacology, University of Bradford, Bradford, West Yorkshire, BD7 1DP

Apomorphine is a potent dopamine receptor agonist (Anden Rubenson, Fuxe & Hokfelt, 1967; Struyker Boudier, 1975), which on intravenous injection in both cats and rats produces a short lasting fall in blood pressure associated with an intense bradycardia (Barnet & Fiore, 1971, Finch & Haeusler, 1973). These effects are centrally mediated, since a pressor response is obtained in spinalized preparations More recently Dutta, Guha & Pradhan (1975) found that intraventricular (i.c.v.) apomorphine produced depressor responses in anaesthetized cats, an effect which was not dose related.

In this study we have examined the effect of i c.v. injections of apomorphine on the resting blood pressure and heart rate in anaesthetized renal hypertensive rats.

Apomorphine (1-100 μg i.c.v.) caused a short lasting dose related fall in blood pressure and heart rate in the urethane-anaesthetized rat preparation. Pretreatment with fluphenazine (0.5 mg/kg i.p.), haloperidol (0.5 mg/kg i.p.) or metoclopramide (10 mg/kg i.p.) abolished the depressor response to apomorphine (10 μg i.c.v.), but pretreatment with piperoxan (200 μg i.c.v. or 10 mg/kg i.p.) did not affect the depressor response to apomorphine (10 μg i.c.v.). Furthermore atropine (1 mg/kg i.p.) pretreatment abolished the cardiovascular effects of apomorphine (10 μg i.c.v.).

Pretreatment 3 days earlier with *p*-chlorophenylalanine (400 mg/kg i.p.), which causes depletion of brain 5-hydroxytryptamine levels (Miller, Cox, Snodgrass & Maickel, 1970), did not affect the depressor response to apomorphine (10 μg i.c.v).

In renal hypertensive rats, intravenous injections of apomorphine (0 01-1 0 mg/kg i.v.) produced short lasting falls in blood pressure associated with an intense bradycardia. Low doses of apomorphine (0.01-0.1 mg/kg i.v.) could be antagonized by pretreatment with haloperidol (0.5 mg/kg i.v.) or metoclopramide (10 mg/kg i.v.). The highest dose was reduced but not abolished.

These results suggest that central dopamine receptors rather than adrenoceptors or serotonin receptors are involved in the mediation of the central hypotensive action of apomorphine in the anaesthetized rat.

A H. is the holder of a S R C. Studentship in conjunction with Dr B.J Alps of Wyeth Laboratories.

References

ANDEN, N E , RUBENSON, A., FUXE, K. & HOKFELT, T (1967). Evidence for dopamine receptor stimulation by apomorphine. *J Pharm. Pharmac.*, 19, 627.

BARNETT, A. & FIORE, J.W. (1971). Hypotensive effects of apomorphine in anaesthetized cats. *Eur J. Pharmac.*, 14, 206-208

DUTTA, S N., GUHA, D & PRADHAN, S N. (1975) Cardiovascular effects of central microinjections of apomorphine in cats. *Arch. int Pharmacodyn.*, **215**, 259-265.

FINCH, L & HAEUSLER, G. (1973). The cardiovascular effects of apomorphine in the anaesthetized rat. *Eur. J. Pharmac.*, 21, 264-270.

MILLER, F.P., COX, R.H., SNODGRASS, W.R. & MAICKEL, R.P. (1970). Comparative effects of *p*-chlorophenylalanine, *p*-chloroamphetamine, *p*-chloro-*N*-methyl-amphetamine on rat brain norepinephrine, serotonin and 5-hydroxyindole-3-acetic acid *Biochem. Pharmac.*, 19, 435-442.

STRUYKER BOUDIER, H. (1975). Two types of dopamine receptors in the brain of the snail Helix aspersa. *Abstracts Sixth Int. Congr Pharmac. Helsinki*, 1975, p. 487.

The interpretation of responses of motoneurone field potentials to 5-hydroxytryptamine

S. BARASI, O. PARRY & M.H.T. ROBERTS*

Department of Physiology, University College, Cardiff

A previous communication to the society (Barasi & Roberts, 1974a) reported that 5-hydroxytryptamine (5-HT) applied by microiontophoresis in the ventral horn of the rat spinal cord, increased the amplitude of antidromically evoked field potential responses of motoneurones. This response to 5-HT closely resembled the effect on motoneurones of stimulating the raphe nuclei. Comparison of these actions of 5-HT with the effect of intravenously administered L-tryptophan on the dorsal root evoked monosynaptic reflex, suggested that 5-HT may, by an unknown mechanism, be increasing the excitability of spinal motoneurones (Barasi & Roberts, 1974b). Several publications, however, have indicated an opposite action of 5-HT (Clineschmidt & Anderson, 1970, Engberg, Flatman & Kadzielawa, 1974) although other reports seem to indicate an increased excitability of motoneurones following administration of monoamines and other drugs (Banna & Anderson, 1968, Marley & Vane, 1967, Sinclair & Sastry, 1974).

In recent studies with the halothane anaesthetized rat conditioning stimulation of nucleus raphes medianus increased lumbar flexor motoneurone field potentials on 22 occasions and had no effect on 10. The extensor field potentials were increased on 16 occasions and unaffected on 4. Iontophoretically applied 5-HT increased flexor potentials on 38 occasions and had no effect on 13. Similarly extensor fields were increased on 32 occasions and unaffected on 8.

To assist with the interpretation of changes in the field potential amplitude, glycine, glutamate and potassium were applied by iontophoresis into the ventral horn. Glycine reduced the amplitude of the field potential on 12 occasions, having no effect on 3. Glutamate increased the potential amplitude on 10 occasions, had no effect on 12 occasions but also reduced the field potential on 8 occasions. Potassium increased the field on 10 occasions in 24 studies but never reduced the amplitude. These observations are compatible with the suggestion that an increased field potential amplitude may be an index of increased motoneurone excitability.

A further test of this postulate involved stimulating the ventral root or muscle nerve (with lumbar dorsal roots sectioned) with 3 to 6 stimuli at 80-200 Hz. The field potential progressively declines in amplitude during the train, presumably due to the failure of propagation of the antidromic spike from axon to soma in a progressively larger proportion of the population of activated motoneurones (Eccles, 1950). 5-HT, glutamate and potassium all tended to prevent this progressive decrease in amplitude. This provides further support for the postulate that motoneurone excitability is increased by these drugs.

This work was supported by a grant from the M R.C.

References

BANNA, N.R. & ANDERSON, E.G. (1968) The effects of 5-hydroxytryptamine antagonists on spinal neuronal activity. *J. Pharmac. exp. Ther.*, **162**, 319-325.

BARASI, S. & ROBERTS, M.H.T (1974a). Motoneurone field potential responses to iontophoretically applied 5-hydroxytryptamine. *Br. J. Pharmac.*, **50**, 446P

BARASI, S. & ROBERTS, M.H T. (1974b). The modification of lumbar motoneurone excitability by stimulation of a putative 5-hydroxytryptamine pathway *Br. J Pharmac.*, **52**, 339-348.

CLINESCHMIDT, B.V & ANDERSON, E.G. (1970). The blockade of bulbospinal inhibition by 5-hydroxytryptamine antagonists. *Exp. Brain Res.*, **11**, 175-186.

ECCLES, J.C. (1950) The responses of motoneurones *Br. med. Bull.*, **6**, 304-311.

ENGBERG, I., FLATMAN, J.A. & KADZIELAWA (1974). The hyperpolarization of motoneurones by electrophoretically applied amines and other agents. *Acta physiol. scand.*, **91**, 3A-4A.

MARLEY, E. & VANE, J.R. (1967). Tryptamine and spinal cord reflexes in cats. *Br. J. Pharmac. Chemother.*, **31**, 447-465.

SINCLAIR, J G. & SASTRY, B S R. (1974). The blockade of bulbospinal inhibition by imipramine, desipramine and parglycine. *Neuropharmacology*, **13**, 643-650.

The selective inhibition of 5-hydroxytryptamine re-uptake by Org 6582

I. GOODLET, S.E. MIREYLEES & M.F. SUGRUE*

Department of Pharmacology, Organon Scientific Development Group, Newhouse, Lanarkshire ML1 5SH

Re-uptake by the membrane pump is considered to be the major means by which monoamines are inactivated at the neural synapse. Numerous studies have shown that tertiary tricyclics, such as chlorimipramine, preferentially inhibit the membrane amine pump of central 5-hydroxytryptaminergic (5-HT) neurones whereas secondary tricyclics, such as desipramine, preferentially block the membrane amine pump of central noradrenergic (NA) neurones (Bopp & Biel, 1974). Several recent reports have emphasized the desirability of compounds exerting a specificity of effect on monoamine re-uptake (Iversen, 1974, van Praag, 1974). The studies to be reported reveal Org 6582 (*dl*-8-chloro-11-anti-amino-benzo-(b)-bicyclo [3.3.1] nona-3,6a (10a) diene hydrochloride) to be a selective inhibitor of 5-HT re-uptake.

In vivo blockade of 5-HT re-uptake was studied by investigating the effect of drug pretreatment on the ability of *p*-chloroamphetamine to lower rat brain 5-HT levels. Intraperitoneally administered Org 6582 was approximately twice as potent as fluoxetine, five times more potent than chlorimipramine and 14 times more potent that desipramine in blocking the ability of *p*-chloroamphetamine to lower brain 5-HT content. Org 6582, whilst having no effect on amine steady-state levels, decreased rat brain 5-HT turnover. Rat brain 5-hydroxyindole acetic acid (5-HIAA) levels were also decreased by Org 6582. The reduction in both 5-HT turnover and 5-HIAA levels is in all probability due to re-uptake blockade.

Peripheral *in vivo* blockade of NA re-uptake was determined by measuring the effect of drug pretreatment on the ability of (−)-metaraminol to lower rat heart NA levels. The i.p. ID_{50} values for desipramine and chlorimipramine were 5.5 mg/kg and 29.6 mg/kg respectively. Org 6582 (60 mg/kg i.p.) did not antagonize the (−)-metaraminol-induced fall in rat heart NA content. Central catecholaminergic re-uptake was studied by investigating the effect of i.p. drug pretreatment on the ability of intraventricularly administered 6-hydroxydopamine (100 μg) to lower rat brain NA and dopamine (DA) levels. The 6-hydroxydopamine-induced fall in rat brain DA content was unaltered by the prior injection of either Org 6582 (60 mg/kg), chlorimipramine (60 mg/kg) or desipramine (30 mg/kg). Pretreatment with either chlorimipramine (60 mg/kg) or desipramine (30 mg/kg), but not with Org 6582 (60 mg/kg), blocked the reduction in brain NA content elicited by 6-hydroxydopamine. Org 6582 had no effect either on steady-state levels or on the turnover of NA and DA in the rat brain.

The results of this study reveal Org 6582 to be a potent selective inhibitor of 5-HT re-uptake.

References

BOPP, B. & BIEL, J H. (1974) Antidepressant drugs. *Life Sci.*, 14, 415-423

IVERSEN, L.L. (1974) Uptake mechanisms for neurotransmitter amines. *Biochem. Pharmac.*, 23, 1927-1935.

VAN PRAAG, H.M (1974) Towards a biochemical typology of depression? *Pharmakopsychiat. Neuro-Psychopharmakol.*, 7, 281-292

Effect of LSD on rat brain 5-hydroxytryptamine metabolism at elevated environmental temperature

G. CURZON & C.A. MARSDEN*

Department of Neurochemistry, Institute of Neurology, Queen Square, London WC1N 3BG

Rat brain tryptophan and 5-hydroxyindoleacetic acid (5-HIAA) concentrations (Tagliamonte, Tagliamonte, Perez-Cruet, Stern & Gessa, 1971) and the firing of 5-hydroxytryptamine (5-HT) containing neurones (Weiss & Aghajanian, 1971) are increased at elevated temperature. The latter two changes appear related, as both are blocked by D-lysergic acid diethylamide (LSD) (Weiss & Aghajanian, 1971) Their relationship to the tryptophan increase is unclear as although this can increase brain 5-HT turnover (Tagliamonte *et al.*, 1971; Knott & Curzon, 1972) additional 5-HT formed is not necessarily released to the synapse. Therefore, we have studied the effects of a 40° C environment on tryptophan disposition and on brain 5-HT metabolism and their modification by LSD.

Male Sprague-Dawley rats (180-200 g) were caged in eight groups of three in a chamber at 25° ± 2° C. Four groups were transferred to a chamber at 40° ± 3° C for 60 min and then killed. LSD tartrate (500 μg/kg i.p.) was given to two of the test groups (40° C) immediately before transfer, and to two control (25° C) groups. Plasma tyrosine, total and free tryptophan and brain tyrosine, tryptophan, 5-HT and 5-HIAA were determined (Curzon, Joseph & Knott, 1972; Knott & Curzon, 1972).

Exposure to 40° C increased plasma total and free tryptophan but not percentage free tryptophan. Brain tryptophan increased markedly, 5-HIAA moderately and 5-HT was not significantly altered (Table 1). Plasma and brain tyrosine were significantly increased (75 and 95% respectively). LSD had no significant effect on either brain or plasma tryptophan, but significantly decreased 5-HIAA (Table 1). The fall was greater at 40° C so that the drug treated rats at both temperatures had similar values.

In another experiment, the rats were given L-tryptophan (50 mg/kg i.p.) 15 min after transfer to the 40° C chamber and then killed 45 min later.

Table 1 Effects of LSD and raised environmental temperature on plasma free and total tryptophan and brain tryptophan, 5-HT and 5-HIAA

	Plasma		*Brain*		
	Total tryptophan *µg/ml*	*Free tryptophan* *µg/ml*	*Tryptophan* *µg/g*	*5-HT* *µg/g*	*5-HIAA* *µg/g*
Control (25°C) (*n* = 6)	13.6 ± 4.0	2.01 ± 0.31	3.02 ± 0.52	0.76 ± 0.04	0.56 ± 0.04
Control + LSD (500 µg/kg i.p.) (*n* = 6)	14.8 ± 2.5	2.43 ± 0.39	3.23 ± 0.36	0.76 ± 0.06	0.48 ± 0.05 *
40°C (*n* = 6)	28.9 ± 3.6 **	4.39 ± 0.60 **	9.04 ± 2.43 **	0.88 ± 0.09	0.72 ± 0.07 **
40°C + LSD (500 µg/kg i.p.) (*n* = 6)	25.3 ± 0.4 **	3.60 ± 0.73 **	7.85 ± 1.16 **	0.83 ± 0.12	0.49 ± 0.04 ***

Rats placed in 40° C environment for 60 min, LSD administered at the start of this period, *n* = no. of rats
Results given as mean ± s.d.
* Significantly different from control (25°C) $P < 0.02$
** Significantly different from control (25°C) $P < 0.01$
*** Significantly different from 40°C $P < 0.01$

Tryptophan increased both brain 5-HT and 5-HIAA. In 40° C LSD pretreated rats, tryptophan caused a significantly smaller 5-HIAA increase, while that of 5-HT tended to be greater.

The results indicate that the increased 5-HIAA in rats exposed to 40° C is not obviously related to increased plasma and brain tryptophan. Also, LSD appeared to decrease catabolism of 5-HT formed from *exogenous* tryptophan at 40° C. If this, like the decreased catabolism of *endogenous* 5-HT after LSD, is caused by decreased release of 5-HT at the synapse, then it implies that exogenous tryptophan can cause increased 5-HT release. Other findings are in agreement (Barasi & Roberts, 1974; Marsden & Curzon, 1975).

This work was supported by an M.R.C. grant.

References

BARASI, S. & ROBERTS, M.H.T. (1974). The modification of lumbar motoneurone excitability by stimulation of a putative 5-hydroxytryptamine pathway. *Br J Pharmac.*, 52, 339-348.

CURZON, G., JOSEPH, M.H. & KNOTT, P.J. (1972) Effects of immobilization and food deprivation on rat brain tryptophan metabolism *J. Neurochem.*, 19, 1967-1974

KNOTT, P.J. & CURZON, G. (1972) Free tryptophan in plasma and brain metabolism. *Nature*, 239, 452-453.

MARSDEN, C.A. & CURZON, G. (1975). Studies on the behavioural effects of tryptophan and para-chlorophenylalanine. *Neuropharmacol.* (in press).

TAGLIAMONTE, A., TAGLIAMONTE, P., PEREZ-CRUET, J., STERN, S. & GESSA, G.L. (1971). Effect of psychotropic drugs on tryptophan concentration in the rat brain. *J. Pharmac. exp. Ther.*, 177, 475-480.

WEISS, B.L. & AGHAJANIAN, G.K. (1971). Activation of brain serotonin metabolism by ttat. Role of midbrain raphe neurons. *Brain Res.*, 26, 37-48.

Alterations in dopamine uptake in rat corpus striatum induced by combinations of stress and Δ^8-tetrahydrocannabinol (Δ^8-THC)

J.M. LITTLETON & K.I. MACLEAN* (introduced by G. BROWNLEE)

Department of Pharmacology, King's College, London WC2

Cannabinoids produce behavioural changes dependent on the environmental conditions under which they are administered (e.g. Carlini, Hamaoui & Martz, 1972). Biochemical changes, also, may be influenced by environment (Littleton & MacLean, 1974). We now report that environment alters the effect of Δ^8-THC on rat striatal dopamine uptake.

Male, Wistar rats, 120-160 g, were kept under 'normal' conditions (housed in groups of four, free access to food and water) for 14 days before every experiment. Rats under 'stressful' conditions were isolated with access to water but not food for 24 h before being killed. Environmental temperature was 21 ± 1°C, the lighting cycle 12 h light (7.00-19.00) and 12 h dark. Animals received Δ^8-THC (10 mg kg^{-1} i.p.) or vehicle (Tween-80, 4% in saline i.p.) 2 h before being killed (11.00-11.30). Uptake of ^{3}H-dopamine into striatum was estimated using the method of Palfreyman, Palfreyman & Clark (1975).

Δ^8-THC pretreatment produced a non-significant reduction in striatal dopamine uptake of rats kept under normal environmental conditions. Striatal preparations from rats kept under stressful conditions showed a small increase in striatal dopamine uptake compared with those from normally-housed rats. Δ^8-THC pretreatment under stressful conditions produced a significant ($P < 0.02$) increase in striatal dopamine uptake compared with preparations obtained from Tween-treated, stressed animals. Under 'normal' conditions, Δ^8-THC produced an uptake of 96.5% ± 12.4%, whilst under 'stressful' conditions Δ^8-THC produced an uptake of 149.1% ± 9.0%, these values being expressed as a percentage ± s.e. mean ($n = 8$) of the appropriate controls.

Previous work on the *in vitro* effect of Δ^8-THC indicates a reduction of dopamine uptake into striatal synaptosomes (Banerjee, Snyder & Mechoulam, 1975). The apparent reversal of this Δ^8-THC-induced change by stress, recalls the *in vitro* findings of List, Bartram, Nazar & Harclerode (1975) that high concentrations of steroids reverse the effect of Δ^9-THC on metabolism in mouse brain homogenates

Our results also explain the previous finding (Littleton & MacLean, 1974) that striatal dopamine depletion after inhibition of dopamine synthesis was reduced by Δ^8-THC only in stressed animals. This increase in dopamine uptake could contribute to the maintenance of dopamine stores and hence reduce the rate of their depletion. In *in vivo* experiments on the conversion of ^{3}H-tyrosine to ^{3}H-dopamine, we have observed no concomitant alteration in striatal dopamine synthesis.

In conclusion, these experiments provide important evidence for the alteration, by stress, of the biochemical effects of Δ^8-THC. When Δ^8-THC is administered in a stressful environment it causes an apparent increase in striatal dopamine uptake. This change implicates central dopaminergic function in the abnormal behaviour patterns induced by Δ^8-THC when administered to stressed animals. The mechanism by which the biochemical effect of Δ^8-THC is altered by environment is currently under investigation.

We wish to thank Beecham Research Laboratories for gifts of Δ^8-THC and radiochemicals In particular, thanks are due to Dr M. Clark and Dr M. Palfreyman for help and advice.

References

BANERJEE, S.P., SNYDER, S.H. & MECHOULAM, R. (1975). Cannabinoids: influence on neurotransmitter uptake in rat brain synaptosomes. *J. Pharmac. exp. Ther.*, **194**, 74-81.

CARLINI, E.A., HAMAOUI, A. & MARTZ, R.M.W. (1972). Factors influencing the aggressiveness elicited by marihuana in food-deprived rats. *Br. J. Pharmac*, **44**, 794-804.

LIST, A.F., BARTRAM, S.F., NAZAR, B.L. & HARCLERODE, J. (1975) Interactions of Δ^9-tetrahydrocannabinol, adrenal steroids and ethanol. *J. Pharm. Pharmac.*, **27**, 606-607.

LITTLETON, J.M & MACLEAN, K.I. (1974). The effect of Δ^8-tetrahydrocannabinol (Δ^8-THC) on dopamine metabolism in the rat corpus striatum: the influence of environment. *Br. J. Pharmac.*, **51**, 117P.

PALFREYMAN, M.G., PALFREYMAN, E.S. & CLARK, M.S.G. (1974). Effects of benapryzine, a new antiparkinson drug, on dopamine uptake into corpus striatum. *Eur. J. Pharmac.*, **28**, 379-383.

CF 25-397 (9, 10-Didehydro-6-methyl-8β-[2-pyridylthiomethyl] ergoline), a new central dopamine receptor agonist

A.L. JATON, D.M. LOEW* & J.M. VIGOURET

Biological and Medical Research Division, Sandoz Ltd., Basle, Switzerland

There is increasing evidence that several ergot derivatives stimulate dopamine receptors in the central nervous system. Bromocriptin (2-Br-α-ergokryptine, CB 154) induces contralateral turning in rats with unilateral lesions in the substantia nigra and produces stereotypies in normal rats. In mice, bromocriptin inhibits reserpine-induced catalepsy and increases motor activity (Johnson, Loew & Vigouret, 1976). CF 25-397 (9,10-Didehydro-6-methyl-8β-[2-pyridylthiomethyl] ergoline) is a new, non-peptide ergot derivative which seems to exert central dopamine-like actions which differ from the effects of bromocriptin or L-DOPA and appear to be more selective.

CF 25-397 induced dose-dependent contralateral turning in rats with 6-hydroxydopamine lesions in the substantia nigra from a dose of 1 mg/kg. However, even at doses 30 times greater, stereotypes were barely detectable (Figure 1). In mice, CF 25-397 inhibited catalepsy induced by reserpine (5 mg/kg i.p., given 17 h before administration of CF 25-397), the ED_{50} for this effect was 0.55 mg/kg s.c. No motor stimulation was observed; on the contrary, CF 25-397 (2.5 to 10 mg/kg s.c.) slightly reduced motor activity.

The fact that the contralateral turning induced by CF 25-397 could be blocked by pimozide (1 mg/kg i.p.) indicates that the compound possesses central dopamine receptor stimulant activities. Unlike other central dopamine receptor agonists, CF 25-397 does not induce stereotypies in the rat. These results suggest that its action on the nigro-striatal system is highly selective. Based on these pharmacological findings it is anticipated that CF 25-397 may provide a new and more selective treatment for patients suffering from extrapyramidal disorders.

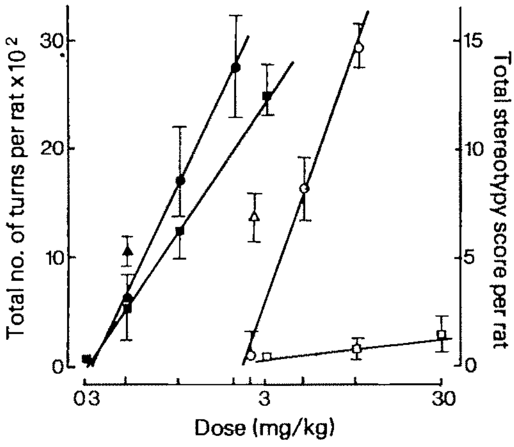


Figure 1 Induction of turning behaviour and stereotypies after subcutaneous injection of CF 25-397, bromocriptin or apomorphine in rats. Solid symbols: means ± s.e. means of the total number of turns in groups of six rats lesioned according to Ungerstedt & Arbuthnott (1970). Open symbols: means ± s.e. mean of the total stereotypy score in groups of six rats, rated according to Costall, Naylor & Olley (1972). (□■) CF 25-397 tartrate; (●○) bromocriptin mesilate; (▲△) apomorphine hydrochloride.

References

JOHNSON, A.M., LOEW, D.M. & VIGOURET, J.M. (1976). Central dopaminergic stimulant properties of bromocriptin in comparison to apomorphine, *d*-amphetamine and L-DOPA. *Br. J. Pharmac.*, 56, (in press).

UNGERSTEDT, U. & ARBUTHNOTT, G.W. (1970). Quantitative recording of rotational behaviour in rats after 6-OH-dopamine lesions of the nigro-striatal dopamine system. *Brain Res.*, 24, 485-493.

COSTALL, B., NAYLOR, R.J. & OLLEY, J.E. (1972). Stereotypic and anti-cataleptic activities of amphetamine after intracerebral injections. *Eur. J. Pharmac.*, 18, 83-94.

The ontogenesis of cerebral dopamine metabolism in the pig

J.P. FRY,* D.F. SHARMAN & D.B. STEPHENS

Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge CB2 4AT

Abnormal oral behaviour, attributed to oral deprivation (Stephens, 1974), can occur in early weaned calves, lambs or pigs. Similar behaviour can be induced in these species by apomorphine which is thought to stimulate cerebral dopamine receptors. Sharman & Stephens (1974) suggested that central dopaminergic neurons might be involved in abnormal oral behaviour such as that seen after early weaning.

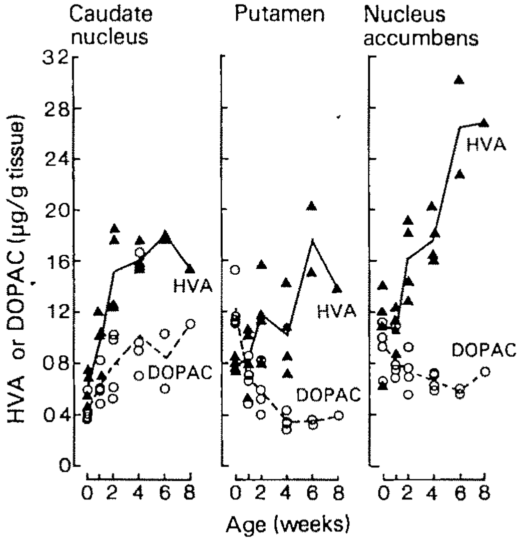


Figure 1 The concentrations of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in different regions of the brain of the pig during the first eight weeks of life. The analyses were made on animals taken from the same two litters over the eight week period

We have estimated the concentrations of dopamine and of its acidic metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in six different parts of the brain of the young pig to obtain an index of the functional activity of the major cerebral dopaminergic neuron systems during the first eight weeks of life. In this way, those regions of the brain showing the most rapid changes in the development of dopaminergic neuronal activity could be determined since these might prove to be of particular importance in the development of abnormal oral behaviour seen after early weaning.

The concentration of dopamine in the caudate nucleus increased greatly and in the putamen showed a small decrease, over the eight week period. In the other regions there was little change in the dopamine concentration. Figure 1 shows the changes in the concentrations of DOPAC and HVA in the three brain regions where large changes in the concentrations of these acidic metabolites occurred. In the caudate nucleus, the concentration of DOPAC appeared to increase as the concentration of HVA increased, whereas in the putamen and nucleus accumbens the concentration of DOPAC was reduced. Some possible implications of these differences will be discussed.

This work was supported in part by a grant from the H.E. Durham Fund, King's College, Cambridge. J.P.F. was supported by an A.R.C. Research Studentship

References

SHARMAN, D.F. & STEPHENS, D.B. (1974) The effect of apomorphine on the behaviour of farm animals. *J. Physiol., Lond.*, 242, 25-27P.

STEPHENS, D.B. (1974). Studies on the effect of social environment on the behaviour and growth rates of artificially-reared British Friesian male calves. *Anim. Prod.*, 18, 23-24

Is metoclopramide a directly acting dopamine receptor antagonist?

I.M. DONALDSON, P. JENNER*, C.D. MARSDEN, R. MILLER & E. PERINGER

University Department of Neurology, Institute of Psychiatry and King's College Hospital Medical School, London SE5 8AF, and M.R.C. Neurochemical Pharmacology Unit, Department of Pharmacology, Medical School, Cambridge CB2 2QD

Metoclopramide produces a blockade of cerebral dopamine (DA) receptors as judged by behavioural (Costall & Naylor, 1973; Dolphin, Jenner, Marsden, Pycock & Tarsy, 1975) and biochemical studies (Ahtee & Buncombe, 1975; Peringer, Jenner & Marsden, 1975). The blockade is presumed to account for the acute dyskinesias produced in man by metoclopramide, by analogy to the classical neuroleptic agents Metoclopramide, however, has no apparent antipsychotic activity suggesting that its mode of action differs from that of neuroleptic drugs.

Administration of metoclopramide (10-100 mg/kg i.p.) to male mice 1.5 h prior to killing produced a similar dose-dependent increase in both striatal and mesolimbic homovanillic acid (HVA) levels. Levels of dihydroxyphenylacetic acid (DOPAC) in striatum and mesolimbic areas were only elevated after metoclopramide 100 mg/kg i.p. Metoclopramide (50 mg/kp i.p.) enhanced the α-methyl-*p*-tyrosine (200 mg/kg 2 h prior to killing; AMPT) induced fall in whole brain DA levels (AMPT treated 385 ± 57 ng/g, metoclopramide treated 171 ± 23 ng/g; $P < 0.005$). Similarly, metoclopramide (50 mg/kg i.p.) enhanced the NSD 1034 (*N*-[3-hydroxybenzyl]-*N*-methylhydrazine dihydrogen phosphate; 150 mg/kg 0.5 h prior to killing) induced accumulation of whole brain DOPA (NSD 1034 treated 496 ± 128 ng/g; metoclopramide treated 1212 ± 182 ng/g; $P < 0.01$). This evidence suggests that metoclopramide increases the synthesis and release of DA. By analogy to the classical neuroleptic drugs, these biochemical changes may be due to functional blockade of cerebral DA receptors suggested by the behavioural studies.

The *in vitro* rat striatal DA sensitive adenylate cyclase system was stimulated approximately two-fold by the addition of DA 10^{-4} M (23.8 to 56.8 pmoles cyclic AMP/2 mg tissue wet wt/2.5 min). Addition of metoclopramide 10^{-7} to 10^{-4} M, however, did not inhibit the stimulation of striatal adenylate cyclase by DA (10^{-4} M).

Ten minutes after injection of metoclopramide (100 μg in 3 μl 0.9% NaCl solution, saline) into one striatum of rats, apomorphine (1 mg/kg i.p.) produced tight circling towards the injected striatum. Injection of 0.9% saline (3 μl), or procaine hydrochloride (100-200 μg in 3 μl 0.9% saline), into one striatum followed by apomorphine (1 mg/kg) was without effect.

This data suggests that the blockade of DA receptors observed following administration of metoclopramide is due to a specific central action of the drug itself, rather than to a peripheral metabolite. However, the failure of metoclopramide to inhibit the DA sensitive adenylate cyclase system *in vitro* suggests a mode of action differing from that of antipsychotic drugs. Whether this represents an action on other DA receptors not tested by the *in vitro* system, or an effect on another neuronal system remains to be determined.

References

AHTEE, L. & BUNCOMBE, G. (1974). Metoclopramide induces catalepsy and increases striatal homovanillic acid content in mice. *Acta Pharmacol. Toxicol.*, 35, 429-432.

COSTALL, B. & NAYLOR, P.J. (1973). Is there a relationship between the involvement of extrapyramidal and mesolimbic brain areas with the cataleptic action of neuroleptic drugs and their clinical antipsychotic effect? *Psychopharmacologia*, 32,

DOLPHIN, A., JENNER, P., MARSDEN, C.D., PYCOCK, C. & TARSY, D. (1975). Pharmacological evidence for cerebral dopamine receptor blockade by metoclopramide. *Psychopharmacologia*, 41, 133-138.

PERINGER, E., JENNER, P. & MARSDEN, C.D. (1975). Effect of metoclopramide on turnover of brain dopamine, noradrenaline and 5-hydroxytryptamine. *J. Pharm. Pharmac.*, 27, 442-444.

Blockade of the electrocortical changes induced by perfusion of amphetamine in the brain stem reticular formation

J.M. CANDY* & B.J. KEY

M.R.C. Neuropharmacology Unit and Department of Pharmacology (Preclinical), The Medical School, Birmingham B15 2TJ

In sleeping cats perfusion of (−)-noradrenaline (NA) or (−)-α-methyl-noradrenaline (MNA) bilaterally into the pontine and mesencephalic reticular formation (MRF) using push-pull cannulae produces short periods of electrocortical desynchronization followed by tonic desynchronization and behavioural arousal. A secondary sedative effect is observed if concentrations in excess of 10^{-3} M are used (Key, 1975).

In the present experiments bilateral perfusion for 5 min of (+)-amphetamine into the MRF of 30 cat *encéphale isolé* preparations mimicked the phasic and tonic electrocortical desynchronization responses induced by NA, but did not produce the secondary sedative effects even when concentrations as high as 10^{-2} M were used. Perfusion of dopamine (10^{-6} to 10^{-3} M) had little or no effect. Concentrations of NA, α-MNA or amphetamine which consistently produced tonic electrocortical desynchronization, were used to study the effects of 6-hydroxydopamine (6-OHDA). Perfusion of 6-OHDA (10^{-4} M) initially produced electrocortical desynchronization which lasted for approximately 30 minutes. After a 1-1.5 h perfusion the electrocortical changes induced by NA or α-MNA (10^{-4} M) were unaltered, but that produced by amphetamine (10^{-3} M) was reduced or abolished, providing the perfusion of amphetamine preceded that of NA or α-MNA. If NA or α-MNA was the first drug tested after 6-OHDA, subsequent perfusion of amphetamine induced electrocortical changes.

These results suggest that amphetamine has a presynaptic action on NA terminals within the MRF and that the NA terminals, although depleted, are still capable of taking up exogenously applied NA or α-MNA after a 1 h perfusion of 6-OHDA. These findings have been further investigated using histochemical methods.

Catecholamine terminals in the MRF of the adult cat are extremely difficult to visualize with the conventional formaldehyde-induced fluorescence method. However, α-MNA is equipotent with NA in its electrocortical effects and since this compound is resistant to monoamine oxidase and also produces a strong fluorophore, it has been used in an attempt to visualize the catecholamine terminals involved in the electrocortical effects produced by amphetamine using the fluorescence method of Falck & Owman (1965). After perfusion of α-MNA (10^{-4} M) for 5 min, catecholamine terminals were observed in the MRF in the vicinity of the cannula tips. The cannula tips were close to the dorsal noradrenergic pathway and uptake of α-MNA was also observed in the axons of this pathway. After perfusion of 6-OHDA (10^{-4} M) for 1 h with prior perfusion of α-MNA, no catecholamine terminals could be observed in the vicinity of the cannula tips. The lack of terminal fluorescence would appear to be due to depletion, since perfusion of artificial CSF for 1 h after a 5 min perfusion of α-MNA did not lead to the disappearance of catecholamine fluorescence. However, after perfusion of 6-OHDA for 1 h followed by perfusion of α-MNA, catecholamine fluorescence, although weak, was still observed in the terminals, indicating that 6-OHDA had not caused complete degeneration in the time interval used. The histochemical data thus support the pharmacological results.

References

FALCK, B. & OWMAN, C. (1965). A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund.*, II, 1-23.

KEY, B.J. (1975). Electrocortical changes induced by perfusion of catecholamines into the brainstem reticular formation. *Neuropharmacology*, 14, 41-51.

Monoamine oxidase active site; the binding to and titration of monoamine oxidase with $[^{14}C]$-selective inhibitors

M.B.H. YOUDIM

M.R.C. Clinical Pharmacology Unit and University Department of Clinical Pharmacology, Radcliffe Infirmary, Oxford OX2 6HE

Liver monoamine oxidase (MAO) (see Youdim, 1975 for review) and the brain enzyme (Salach, Yasunobu, Minamura & Youdim, 1975) contain 1 mol of covalently bound flavin adenine dinucleotide (FAD) as a cofactor. The isolated FAD is associated with a peptide, having the following amino acid sequence, Ser-Gly-Gly-Cys-Tyr, the flavin being attached via the 8α-carbon of riboflavin in a thio-ether linkage with the cysteine residue (Salach *et al.*, 1975).

Enzyme absorption spectra studies with selective $[^{14}C]$-labelled irreversible inhibitors have shown that the action of clorgyline (*N*-methyl-*N*-propargyl-3-(2,4-dichlorophenoxy) propylamine hydrochloride), deprenil (phenyl-isopropylmethyl-propinylamine hydrochloride) and phenylethylhydrazine (Collins & Youdim, 1975) may involve the binding of the inhibitors with the flavin cofactor. The inhibition of enzyme by these compounds is prevented by monoamine substrates, is time-dependent and is a function of the amount of inhibitor bound to the enzyme. When MAO is fully inhibited 1 mol of the inhibitor deprenil or phenylethylhydrazine combines irreversibly and covalently to 1 g equivalent of purified enzyme of molecular weight 120,000-150,000 (Youdim & Sourkes, 1966) in a stoichiometric fashion.

Isolation of the flavin peptide adduct from $[^{14}C]$-inhibitor treated liver enzyme showed that 1 mol of $[^{14}C]$-inhibitor is bound to 1 mol of flavin. Fluorescence and spectral absorption studies suggest that deprenil may bind to the nitrogen on the flavin at position 5, a result which is similar to that reported for the inhibitor 3-dimethylamino-1-propyne (Maycock, Abeles, Salach & Singer, 1975).

Certain physiological factors influence the activity of MAO, which in turn may affect monoamine metabolism. The changes in enzyme activity could be due to a change in MAO protein synthesis or degradation. In experimental studies and disease states in the human where MAO activity fluctuates it is desirable to know the exact amount of enzyme present. The results presented in this communication suggest the use of labelled selective inhibitors as agents for titration of MAO and its multiple forms because the inhibition is stoichiometric and thus the titration end-point can be used to estimate the enzyme concentration.

References

COLLINS, G.G.S. & YOUDIM, M.B.H (1975) The binding of (^{14}C)-phenylethylhydrazine to rat liver monoamine oxidase. *Biochem. Pharmac.*, **24**, 703-706

MAYCOCK, A.L., ABELES, R.H., SALACH, J.I. & SINGER, T.P. (1975). Structure of the adduct derived from the inhibition of monoamine oxidase by 3-dimethylamino-1-propyne In *Flavins and Flavoproteins*, ed. T.P. Singer. Amsterdam. Elsevier, (in press)

SALACH, J.I., YASHUNOBU, K.T., MINAMURA, M. & YOUDIM, M.B.H. (1975). Is there covalently bound flavin in monoamine oxidase from brain? In *Flavins and Flavoproteins*, ed. T.P. Singer. Amsterdam· Elsevier, (in press).

YOUDIM, M.B.H. (1975). Monoamine deaminating system in mammalian tissues. In *M.T.P. International Review of Science*, ed. H. Blaschko, pp. 169-211. Oxford Press: Butterworth Ltd.

YOUDIM, M.B.H. & SOURKES, T.L. (1966) Properties of purified, soluble monoamine oxidase. *Can. J. Biochem.*, **44**, 1397-1400.

Influence of etorphine acepromazine and diprenorphine on cardiovascular function in ponies

C.J. HILLIDGE & P. LEES*

Department of Physiology, The Royal Veterinary College, Hawkshead Lane, North Mymms, Hatfield, Herts, AL9 7TA

The neuroleptanalgesic drug combination of etorphine and acepromazine (Large Animal Immobilon; Reckitt & Colman Ltd.) was administered i.v. at the recommended dose rate (24 μg/kg etorphine and 100 μg/kg acepromazine) to twelve Welsh Mountain ponies of 185 to 336 kg bodyweight. Cardiovascular measurements were made before and at pre-determined times up to 30 min after the injection. The etorphine antagonist, diprenorphine (Revivon; Reckitt & Colman Ltd.), was then injected i.v. (30 μg/kg) and further measurements were obtained.

Pronounced increases in heart rate, moderate increases in cardiac output and significant reductions in stroke volume occurred throughout the period of neuroleptanalgesia (Table 1). Mean

Table 1 Effects of etorphine, acepromazine and diprenorphine on cardiovascular function

Time (min)	*Heart rate (beats/min)*	*Stroke volume (ml kg^{-1})*	*Cardiac output (ml kg^{-1} min^{-1})*	*TPR (dyn s cm^{-5})*	*MAP (mm Hg)*	*Hb concentration (g/100 ml)*
control	42 ± 2	1.66 ± 0.07	66 ± 3	656 ± 41	123 ± 3	11.7 ± 0.3
E + 5	134 ± 10**	0.77 ± 0.07**	102 ± 13**	608 ± 65	161 ± 8**	14.5 ± 0.3**
E + 15	117 ± 8**	0.76 ± 0.04**	87 ± 6**	474 ± 44**	117 ± 7	13.3 ± 0.3**
E + 30	122 ± 12**	0.83 ± 0.11**	93 ± 7**	503 ± 62**	126 ± 6	12.7 ± 0.3
D + 5	93 ± 12**	1.03 ± 0.07**	91 ± 10*	426 ± 60**	96 ± 4**	12.0 ± 0.4
D + 15	66 ± 6**	1.23 ± 0.08**	77 ± 6	434 ± 48**	90 ± 4**	10.7 ± 0.3**
D + 30	52 ± 4*	1.32 ± 0.07**	66 ± 5	546 ± 59*	90 ± 5**	9.8 ± 0.2**
D + 60	47 ± 3	1.37 ± 0.07**	62 ± 3	539 ± 50**	93 ± 4**	9.3 ± 0.2**

Values are means ± s.e. mean for twelve ponies. Times refer to the i.v. administration of etorphine and acepromazine (*E*) or the i.v. injection of diprenorphine (*D*). The significance of differences from control values was assessed by paired *t*-tests and is indicated by asterisks: *, $P < 0.05$, **, $P < 0.01$.

arterial blood pressure (MAP) and arterial HB concentration were increased after 5 min but had returned to the control ranges by 15 min (MAP) and 30 min (Hb concentration). Total peripheral resistance (TPR) was initially unchanged and then reduced at 15 and 30 minutes. Following diprenorphine administration, TPR decreased further and MAP and Hb concentration also fell below control levels, while heart rate and cardiac output initially remained above controls before returning to the normal ranges by 60 and 15 min, respectively.

It is concluded that etorphine causes sympathoadrenal stimulation as a result of its respiratory depressant effects (Hillidge & Lees, 1975) and possibly by other mechanisms and that some of the sympathetic effects are partially offset by an α-adrenoceptor blocking action of acepromazine. This action of acepromazine probably accounted also for the reductions in MAP, TPR and Hb concentration which occurred after the actions of etorphine had been antagonized with diprenorphine.

This study was supported by the Wellcome Foundation and the Horserace Betting Levy Board. Drugs were supplied by Reckitt and Colman Ltd. F.C. Allsup and J. Millar provided skilled technical assistance.

References

HILLIDGE, C.J. & LEES, P. (1975). Influence of the neuroleptanalgesic combination of etorphine and acepromazine on the horse: blood gases and acid-base balance. *Equine vet. J.*, , 7, 148-154.

The effect of inhibitors of alcohol metabolism on the changes in the hepatic microsomal metabolism of foreign compounds produced by a single dose of ethanol in the rat

L. GRANT & G. POWIS*

Department of Pharmacology, Glasgow University, Glasgow G12 8QQ, Scotland

•

The administration to rats of a single oral dose of ethanol has been found to produce a selective induction of hepatic microsomal aniline hydroxylation, a decrease in aminopyrine demethylation and no change in the activity of the components of the hepatic microsomal mixed function oxidase (Powis, 1975). The present work is an attempt to distinguish between the direct effects of ethanol and the effects resulting from the metabolism of the ethanol, on the hepatic microsomal metabolism of foreign compounds.

Ethanol or acetaldehyde were administered to unanaesthetized rats by stomach tube and inhibitors of alcohol and aldehyde dehydrogenase were administered by i.p. injection. The hepatic microsomal fraction was prepared, 24 h after the administration of the various compounds, by the method of Ernster, Siekevitz & Palade (1962) in 0.25 M sucrose containing 0.05 M Tris buffer pH 7.4. The metabolism of foreign compounds was measured over 30 min at 37°C using the incubation conditions described by Mazel (1971). The formation of *p*-aminophenol from aniline was measured by the method of Schenkman, Remmer & Estabrook (1967) and formaldehyde formed from aminopyrine by the method of Nash (1953). Microsomal protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

Acetaldehyde oxime (200 mg/kg), an inhibitor of alcohol dehydrogenase (Lester & Benson, 1970), itself produced no significant change in aniline hydroxylation but caused an increase in aniline hydroxylation in rats fed ethanol (85 mmol/kg) from 9.4 ± 0.4 (mean ± s.e. mean) to 21.9 ± 1.3 nmol mg^{-1} 30 min^{-1} ($n = 6$, $P < 0.01$). Acetaldehyde oxime produced a decrease in aminopyrine demethylation of a similar magnitude to that caused by ethanol itself although there was no additive effect when both compounds were administered together. Pyrazole (200 mg/kg), also an inhibitor of alcohol dehydrogenase (Goldberg & Rydberg, 1969), produced an increase in aniline hydroxylation and a decrease in aminopyrine demethylation. It had similar effects to acetaldehyde oxime, however, on the changes in metabolism produced by ethanol.

Acetaldehyde (22 mmol/kg, fed to rats 24 h and 18 h before the preparation of the microsomal fraction) had no effect upon aniline hydroxylation but produced a decrease in aminopyrine demethylation from 105.3 ± 5.8 to 75.5 ± 5.3 nmol mg^{-1} 30 min^{-1} ($n = 6$, $P < 0.01$). Disulphiram (300 mg/kg), an inhibitor of aldehyde dehydrogenase (Dietrich & Erwin, 1971), reduced aminopyrine demethylation to 70.9 ± 3.6 nmol mg^{-1} 30 min^{-1} ($n = 6$, $P < 0.01$) but also potentiated the decrease in aminopyrine demethylation in rats fed only half the previous dose of acetaldehyde to 37.9 ± 5.1 nmol mg^{-1} 30 min^{-1} ($n = 6, P < 0.05$).

It is concluded from these results that the increase in aniline hydroxylation produced by the administration of a single dose of ethanol is a consequence of the ethanol itself, whilst the decrease in aminopyrine demethylation is a consequence of the metabolism of ethanol to acetaldehyde.

References

DIETRICH, R.A. & ERWIN, V.G. (1971). Mechanism of the inhibition of aldehyde dehydrogenase *in vivo* by disulfiram and diethyldithiocarbamate. *Molec. Pharmac.*, 7, 301-307.

ERNSTER, L., SIEKEVITZ, P. & PALADE, G E. (1962). Enzyme structure relationships in the endoplasmic reticulum of rat liver. *J cell Biol.*, **15**, 541-562.

GOLDBERG, L. & RYDBERG, U. (1969). Inhibition of ethanol metabolism *in vivo* by administration of pyrazole. *Biochem. Pharmac.*, 18, 1749-1762.

LESTER, D. & BENSON, G.D. (1970). Alcohol oxidation in rats inhibited by pyrazole, oximes and amides. *Science, N.Y.*, **169**, 282-284.

LOWRY, O H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193**, 265-275.

MAZEL, P. (1971). Experiments illustrating drug metabolism *in vitro*, in *Fundamentals of Drug Metabolism and Drug Disposition*, ed. LaDu, B,N., Mandel, H.G. & Way, E L. p. 546-582. Baltimore: Williams Wilkins Co.

NASH, T. (1953). The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.*, 55, 416-421.

POWIS, G. (1975). Effect of a single oral dose of methanol, ethanol and propan-2-ol ,on the hepatic microsomal metabolism of foreign compounds in the rat. *Biochem. J.*, **148**, 269-277.

SCHENKMAN, J.B., REMMER, H. & ESTABROOK, R.W. (1967). Spectral studies of drug interaction with hepatic microsomal cytochrome. *Molec. Pharmac.* **3**, 113-123.

Catecholamine metabolism and liver dysfunction during induction of ethanol dependence

C. ABU-MURAD, P.J. GRIFFITHS & J.M. LITTLETON (introduced by G. BROWNLEE)

Department of Pharmacology, King's College, London

We have reported that concentrations of noradrenaline and dopamine increase in brain during the induction of ethanol dependence in mice (Griffiths, Littleton & Ortiz, 1974). These changes can be demonstrated to be an important factor in the induction of dependence, and their cause is of interest. The experiments reported here suggest a possible mechanism.

We have induced ethanol dependence in TO strain, white mice as described previously (Griffiths *et al.*, 1974). Mice were killed at intervals during chronic ethanol administration by whole body immersion in liquid nitrogen, brain concentrations of amino acids estimated (modified gas-liquid chromatographic method of Islam and Darbre, 1969) and livers taken for estimation of triglycerides (methods of Folch, Lees & Sloane Stanley, 1957; Van Handel & Zilversmit, 1957). In parallel experiments mice were withdrawn daily from ethanol for assessment of the ethanol withdrawal syndrome.

The significant changes in several central amino acid concentrations which occur, will be described in detail elsewhere. In the context of this communication, the most interesting finding was that the concentration of tyrosine in brain showed a steady rise during ethanol administration with a time course which preceded that of the catecholamine accumulation reported previously. Since Wurtman, Larin, Mostafapour & Fernstrom (1974) have shown that brain tyrosine concentration directly influences the synthesis of catecholamines, it seems likely that the cause of catecholamine accumulation may be the increased concentration of tyrosine. The source of this increase in tyrosine is unknown but the second part of this investigation suggests that it may be a consequence of liver dysfunction induced by ethanol administration.

Liver triglyceride accumulation showed a striking similarity in time course with the intensity and duration of the withdrawal syndrome. About 6 days of ethanol administration are required before significant changes occur, the changes reach a maximum after 8 or 9 days. In female mice hepatic triglyceride accumulation is slower and these animals are correspondingly resistent to induction of dependence. This somewhat unexpected evidence that liver dysfunction may be directly related to the induction of ethanol dependence, may be linked with the changes in catecholamine metabolism discussed earlier. Preliminary experiments suggest that livers showing evidence of triglyceride accumulation have a reduced capacity for uptake of monoamine precursors.

In conclusion, we believe that liver dysfunction may influence central catecholamine metabolism by an alteration in the handling of catecholamine precursors. Such a mechanism has already been proposed (Knell, Davidson, Williams, Kantameni & Curzon, 1974) to explain changes observed in hepatic encephalopathy We suggest that functional liver damage is not merely a secondary pathological finding associated with alcoholism but that it contributes directly to the central disease process.

References

FOLCH, J., LEES, M. & SLOANE STANLEY G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. biol Chem.*, **226**, 497-509

GRIFFITHS, P.J., LITTLETON, J.M. & ORTIZ, A (1974). Changes in monoamine concentrations in mouse brain associated with ethanol dependence and withdrawal. *Br. J. Pharmac.*, **50**, 489-498.

ISLAM, A. & DARBRE, A. (1969). Gas liquid chromatography of trifluoroacetylated amino acid methyl esters. Development of a mixed stationary phase for their reparation. *J. Chromatogr.*, **43**, 11-24.

KNELL, A.J., DAVIDSON, A.R., WILLIAMS, R., KANTAMENI, B.D. & CURZON, G. (1974). Dopamine and Serotonin metabolism in hepatic encephalopathy. *Br. med. J.*, **15**, 495-551.

VAN HANDEL, E. & ZILVERSMIT, D.B. (1957). Micromethod for the direct determination of serum triglycerides. *J. Lab. clin. Med.*, **50**, 152-157.

WURTMAN, R J., LARIN, F., MOSTAFAPOUR, S. & FERNSTROM, J.D (1974). Brain catechol synthesis, control by brain tyrosine concentration. *Science, N.Y.*, **185**, 183-184.

Are central cholinergic pathways involved in the habituation of exploration and distraction?

SANDRA E. FILE (introduced by M.J. NEAL)

Department of Pharmacology, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX

Carlton (1968) has suggested that a central muscarinic cholinergic system is essential for habituation of exploration. He exposed rats injected with scopolamine to a novel environment, but based his conclusions on their performance the following day when they were untreated. The results could be due to state-dependent learning (Overton, 1966) rather than to scopolamine preventing habituation. The purpose of the present study was to test whether muscarinic systems in the brain are involved in long-term habituation of exploration and distraction.

The effects of three muscarinic antagonists on habituation of exploration were tested in a holeboard with four holes, under which objects could be placed (File & Wardill, 1975). Exploration was measured by the frequency (head-dips/10 min) and duration of head-dipping(s). Ten minute trials were given, separated by 24 hours.

As illustrated in Figure 1, when objects were present, scopolamine hydrobromide (1 & 2 mg/kg i.p.) increased exploration in rats ($P < 0.01$) and the duration of head-dipping habituated over trials

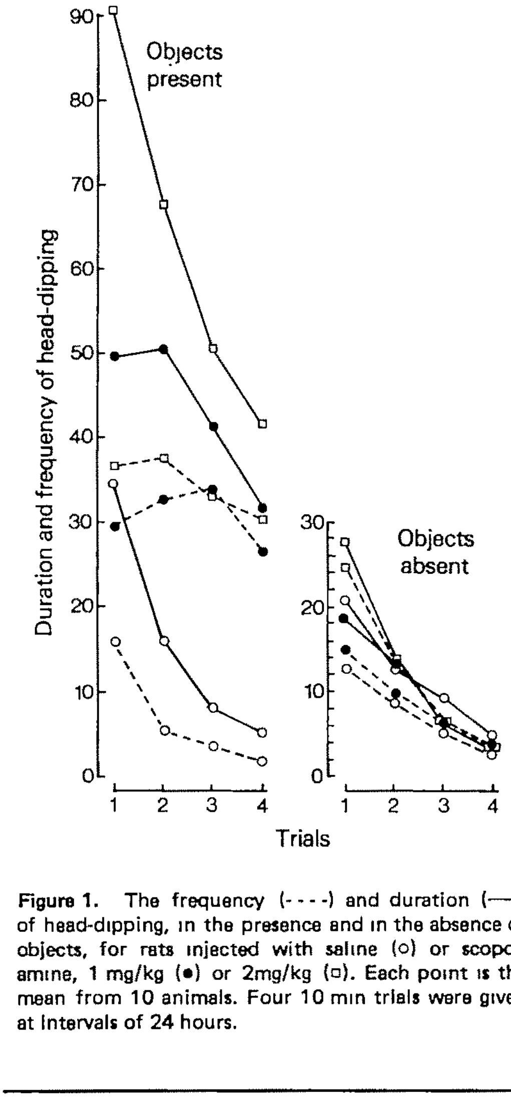


Figure 1. The frequency (- - - -) and duration (——) of head-dipping, in the presence and in the absence of objects, for rats injected with saline (○) or scopolamine, 1 mg/kg (●) or 2mg/kg (□). Each point is the mean from 10 animals. Four 10 min trials were given at intervals of 24 hours.

($P < 0.001$). The frequency of head-dipping did not habituate in the scopolamine groups. No drug effects were found when rats were tested in the absence of objects, i.e in a simpler task. A similar pattern of results was shown by mice. Neither atropine sulphate (10 and 20 mg/kg i.p.) nor benzhexol hydrochloride (40 mg/kg i.p.) affected habituation of exploration in rats, whether this was measured by the frequency or the duration of head-dipping, and regardless of whether objects were present or absent.

The effects of muscarinic antagonists on habituation of distraction were also studied. Tones were presented to rats and their distraction and subsequent habituation to these measured by the interruption in their base-line licking. Scopolamine, methylscopolamine, atropine, methylatropine and benzhexol all failed to impair habituation.

These experiments provide no support for the suggestion that central muscarinic systems are essential for habituation of exploration or distraction.

This work was supported by a Roche Research Fellowship to S.E.F. and a Science Research Council grant to Dr M.J. Neal.

References

CARLTON, P.L. (1968). Brain-Acetylcholine and habituation. *Progress in Brain Research, Anticholinergic Drugs and Brain Functions in Animals and Man*, Bradley, P.B. & Fink, M. eds., Amsterdam: Elsevier, 28, 48-60.

FILE, S.E. & WARDILL, A.G. (1975). Validity of head-dipping as a measure of exploration in a modified holeboard. *Psychopharmacologia*, 44, 53-59

OVERTON, D.A. (1966). State-dependent learning produced by depressant and atropine-like drugs. *Psychopharmacologia*, 10, 6-31.

Effect of some phospholipids on acetylcholine output from the cerebral cortex in the rat

L. AMADUCCI, P. MANTOVANI & G. PEPEU*

Departments of Pharmacology and Neurology, University of Florence Medical School, Florence, Italy

The effect of phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) on acetylcholine (ACh) output from the cerebral cortex was investigated in adult Wistar rats under urethane anaesthesia according to the procedure described by Hemsworth & Neal (1968). ACh output was determined by placing a collecting cylinder filled with eserinized Ringer solution on the exposed frontoparietal cortex. Every 10 min the solution was changed and bioassayed on the dorsal muscle of the leech. The phospholipids were prepared from bovine brain and we checked their purity by thin layer chromatography. They were injected intravenously as a sonicated suspension in Tris buffer in a volume not exceeding 0.3 ml.

The spontaneous ACh output from the cerebral cortex prior to the phospholipid administration was 1.63 ± 0.18 ng.cm^{-2} min^{-1} ($n = 36$). PS injected at the doses of 50, 75, and 150 mg/kg caused increases in ACh output of 57%, 83% and 313% respectively. PE, 150 mg/kg, only caused a 50% increase and PC had no effect. The increase only occurred in the two samples collected following the phospholipid administration. When calcium was removed from the Ringer solution and substituted with magnesium the spontaneous ACh output was decreased and the increase induced by PS was either prevented or strongly reduced.

It has been shown by Mongar & Svec (1972) that PS potentiates the histamine release by antigen from three sensitized rat tissues. This effect is calcium dependent. It therefore seems possible that a number of secretory processes at peripheral and central levels might be affected by PS through a mechanism in which calcium plays an important role.

We thank Fidia Laboratories Abano Terme, Italy for the supply of purified phospholipids. This work was supported by CNR grant No. 74.00250.04.

References

HEMSWORTH, B.A. & NEAL, M.J. (1968) The effect of central stimulant drugs on the release of acetylcholine from the cerebral cortex. *Br. J. Pharmac.*, 32, 543-550.

MONGAR, J.L. & SVEC, P. (1972). The effect of phospholipids on anaphylactic histamine release. *Br. J. Pharmac.*, 46, 741-752.

Developmental changes in the sensitivity of neurohormone-stimulated cyclic AMP formation in chick cerebral hemispheres

S.R. NAHORSKI, WENDY PATTON* & K.J. ROGERS

Department of Pharmacology, University of Sheffield

In a previous study from this laboratory we reported that in the *in vivo* 3′-5′-adenosine cyclic monophosphate (cyclic AMP) content of chick cerebral hemispheres decreased with increasing age (Nahorski, Rees & Rogers, 1975). Moreover, whereas the responsiveness of the cerebral cyclic AMP system to isoprenaline and histamine *in vivo* fell throughout the post-natal period, the post mortem ischaemic increase in the cyclic nucleotide became progressively larger with age. In the present experiments we have used *in vitro* techniques in an attempt to elucidate possible mechanisms for these developmental changes in sensitivity.

Experiments were performed on Rhode Island Red X Sussex Brown chicks and embryos at various stages during their perinatal development. Cerebral hemisphere slices (0.37 mm thick) were preincubated for 60 min in a Krebs-bicarbonate buffer containing 10 mM glucose and following exposure of the slices to various agonists, the cyclic AMP content of the tissue was determined by a protein-binding assay. Responsiveness to isoprenaline and histamine in the slices became evident at 17 days embryonic age whereas a response to adenosine was observed at 15 days embryonic age. By means of dose-response curves it was found that the maximal cyclic AMP response to isoprenaline (493 p moles/mg protein) and histamine (524 p moles/mg protein) occurred at 3 days postnatal age and then declined during the first postnatal month. The largest increase in the nucleotide concentration following exposure to adenosine was observed at 19 days embryonic age (211 p moles/mg protein) and this response was maintained throughout the period studied. Despite the apparent developmental alterations in the maximum response to different agonists, the ED_{50} for each of the agonists was similar at all ages studied.

The activity of adenylate cyclase in cerebral homogenates of chicks of various ages was assayed in the presence and absence of 10 mM NaF. Significant basal activity was observed in 15 day old embryos and this increased at least 4 fold by 21 days postnatal age. NaF only significantly stimulated enzyme activity in tissue from the oldest birds. Ethyleneglycolbis (β-aminoethyl)-*N*, *N*′-tetracetic acid (EGTA) markedly suppressed enzyme activity and in the presence of this Ca^{++} chelator no developmental changes in adenylate cyclase activity were observed. Phosphodiesterase activity was determined using low (1 μM cyclic AMP) substrate concentrations. Enzyme activity increased from 949 ± 82 p moles cyclic AMP hydrolysed min^{-1} mg^{-1} protein at 15 days embryonic age to 4529 ± 153 p moles cyclic AMP hydrolysed min^{-1} mg^{-1} protein at 4 weeks post hatch.

The results suggest that in the embryonic and neonatal periods the development of cerebral neurohormone receptors coupled to adenylate cyclase may precede the development of functional transmitter input leading to "supersensitive" responses to exogenous agonists. This resembles the supersensitivity demonstrated in cerebral tissue to isoprenaline after chemical denervation. (Nahorski & Rogers, 1975).

Financial support from the Medical Research Council is gratefully acknowledged.

References

NAHORSKI, S.R., REES, M.W. & ROGERS, K.J. (1975). Cyclic AMP in developing chick brain: changes with ischaemia and catecholamine administration. *Br. J. Pharmac.*, 54, 255-256P.

NAHORSKI, S.R. & ROGERS, K.J. (1975). Altered sensitivity of β-adrenoceptor-mediated cyclic AMP formation in brain. *Br. J. Pharmac.* (in press).

A primate model of acute dystonic reaction to neuroleptics

G.M. ANLEZARK*, C.D. MARSDEN & B.S. MELDRUM

Department of Neurology, Institute of Psychiatry, London, SE5 8AF

A small proportion of patients (5% or less) treated with classical neuroleptic drugs of the butyrophenone and phenothiazine series develop acute dystonic reactions in the initial few days of therapy. The mechanism by which these reactions are produced is unknown, but the antipsychotic action of neuroleptics is thought to be due to blockade of central dopamine synapses.

In the course of testing the neuroleptics haloperidol (0.6-1.2 mg/kg i.v.) and pimozide (0.1-2.5 mg/kg i.v.) in the photosensitive Senegalese baboon, *Papio papio*, we discovered that three animals showed a dystonic response (Meldrum, Anlezark & Trimble, 1975). One animal showed the abnormal response only after the highest dose of pimozide and was not used in the subsequent experiments. The other two invariably showed the response with all doses tested. The normal response to neuroleptics in these baboons was sedation and a reduction in spontaneous motor behaviour lasting up to 5 hours. The abnormal response was characterized by episodes of compulsive gnawing, tongue protrusion, neck extension and trunk twisting and by licking accompanied by salivation and hyperventilation lasting up to 7 hours. This dystonic reaction was also produced by chlorpromazine (5 and 25 mg/kg i.m.), but not by thioridazine (3 and 7 mg/kg i.v.). It was abolished by the anticholinergics benztropine (0.2 mg/kg i.v.) and hyoscine (20 and 50 µg/kg i.v.). This is analogous to the situation in human patients, in whom anticholinergic drugs dramatically abolish acute dystonic reactions to neuroleptics. Thioridazine is a potent antipsychotic which has few extrapyramidal side effects, perhaps because it possesses high inherent anti-muscarinic activity (Miller & Hiley, 1974).

In acute experiments, the neuroleptics cause increased turnover and release of dopamine in the rat striatum (Andén, Butcher, Corrodi, Fuxe & Ungerstedt, 1970), and dystonic postures and movements are provoked by L-DOPA in some patients with Parkinson's disease. A possible explanation of dystonic reactions to neuroleptic drugs is that the dopamine release that they cause activates dopaminergic synapses not blocked by the neuroleptics. If this is so, depletion of central dopamine by reserpine or the tyrosine hydroxylase inhibitor, α-methyl-p-tyrosine (AMPT) should reduce or abolish the abnormal responses. Pretreatment with AMPT alone (200 mg/kg i.p. in a single dose or 150 mg/kg i.p x 3) had little effect on the dystonic response to haloperidol (1 mg/kg i.v.), but pretreatment with reserpine (2 mg/kg i.p.) and AMPT (200 mg/kg i.p. or 150 mg/kg i.p. x 2) decreased the severity of the dystonic response to haloperidol and delayed its onset.

References

ANDÉN, N-E., BUTCHER, H., CORRODI, H., FUXE, K. & UNGERSTEDT, U. (1970). Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *Eur. J. Pharmac.*, 19, 303-314.

MELDRUM, B.S., ANLEZARK, G.M. & TRIMBLE, M. (1975). Drugs modifying dopaminergic activity and behaviour, the EEG and epilepsy in *Papio papio*. *Eur. J. Pharmac.*, 32, 203-213.

MILLER, R.J. & HILEY, C.R. (1974). Anti-muscarinic properties of neuroleptics and drug-induced Parkinsonism. *Nature, Lond.*, 248, 596-597.

Stereospecific suppression by opiates of the quasi-morphine abstinence syndrome elicited by 3-isobutyl-l-methylxanthine (IBMX)

H.O.J. COLLIER & D.L. FRANCIS*

Research Department, Miles Laboratories Limited, Stoke Poges, Slough

Methylxanthines elicit from otherwise untreated rats several of the behavioural signs seen in the morphine abstinence syndrome (MAS). Heroin readily suppresses the signs elicited by methylxanthines, and naloxone increases their intensity and elicits other signs, such as jumping, to produce a behavioural pattern scarcely distinguishable from the MAS (Collier, Francis, Henderson & Schneider, 1974; Francis, Roy & Collier, 1975). We have, therefore, called the behaviour elicited by xanthines the quasi-morphine abstinence syndrome (QMAS). The present work examines whether suppression by opiates of the QMAS is stereospecific and whether drugs modifying it are effective when injected into a cerebral ventricle.

Drugs were injected into male white Wistar rats (110-200 g) either subcutaneously (s.c.) or, through an indwelling cannula, into the left lateral cerebral ventricle (i.c.v.). The QMAS was elicited with 3-isobutyl-1-methylxanthine (IBMX, 15 mg/kg s c.) and effects on behaviour were observed 'blind' for up to 15 min after treatment with coded solutions. Fourteen behavioural signs –jumping, teeth chattering, squeak on touch, squeak on handling, diarrhoea, chewing, ptosis, body shakes, head shakes, paw tremor, rearing, restlessness, salivation and licking the penis–were recorded. Total 'quasi-abstinence score' was obtained by counting 1 for presence and 0 for absence of each sign, and was expressed as a median value. The significance of differences between scores was determined by the Mann-Whitney U test.

Heroin (30-300 μg/kg s.c. or 1 and 10 μg/rat i.c.v.) overcame the QMAS in a dose-related way. When the QMAS had been suppressed with heroin (300 μg/kg s.c.), naloxone (10-100 μg/kg s.c. or 0.1-10 μg/rat i.c.v) reversed the suppression in a dose-related way. Levorphanol (10-100 μg/kg s.c.) also suppressed the QMAS, but dextrorphan was ineffective at 8 mg/kg s c. These effects were statistically significant at P values ranging downwards from $P = 0.027$ to $P < 0.001$.

That the potent effect of opiates in overcoming the QMAS due to IBMX is stereospecific and is reversed by very small doses of naloxone shows that this is an opiate agonist action. Methylxanthines in turn antagonize opiate agonist actions (Bellville, 1964; Ho, Loh & Way, 1973). This mutual antagonism may provide a clue to the mechanisms of action of both types of drug and to the mechanisms of dependence.

These findings also offer a sensitive *in vivo* method of detecting and estimating the behavioural effects of morphine-like, methylxanthine-like or naloxone-like substances.

We thank Mr N.J. Cuthbert and Miss J.F. de C Sutherland for technical help, Roche Products Limited for levorphanol and dextrorphan, and Sankyo Limited for naloxone.

References

BELLVILLE, J.W. (1964). Interaction of caffeine and morphine on respiration. *J. Pharmac. exp. Ther.*, **143**, 165-168.

COLLIER, H O.J., FRANCIS, D.L., HENDERSON, G. & SCHNEIDER, C. (1974). Quasi-morphine abstinence syndrome. *Nature, Lond.*, **249**, 471-473.

FRANCIS, D.L., ROY, A.C. & COLLIER, H.O.J. (1975). Morphine abstinence and quasi-abstinence effects after phosphodiesterase inhibitors and naloxone. *Life Sci.*, **16**, 1901-1906.

HO, I.K , LOH, H.H. & LEONG WAY, E. (1973). Effects of cyclic 3',5'-adenosine monophosphate on morphine tolerance and physical dependence. *J. Pharmac. exp. Ther.*, **185**, 346-357.

Physiological aspects of the hypnotic properties of steroid hormones

MARGARETHE HOLZBAUER

Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge CB2 4AT

Although it has been known for nearly half a century that some biogenic steroids possess hypnotic potencies the physiological significance of this effect is still debated. Research in this field was mainly directed towards the development of anaesthetic agents for clinical use and a large number of steroids have been tested for their central depressant activities. They were low in those steroids with high conventional "hormonal activities". However they were very high in some of the hepatic catabolites of steroid hormones which are reduced in ring A and are deprived of their original "hormonal activities". Some of these compounds, e.g., pregnanolone (a metabolite of progesterone) or the ring A-reduced form of deoxycorticosterone were in some tests up to ten times more active than barbiturates. In the liver, these metabolites are however also esterified, become less lipid soluble and lose hypnotic activity. Interest in the physiology of the hypnotic properties of steroids was revived when it was found that they can be produced and secreted into the blood stream at sites from where they can reach the brain without having to pass first through the liver. For example, the ovary of the rat can produce several ring A-reduced steroids in quantities similar to or even larger than those of progesterone (Holzbauer, 1969, 1971a, Holzbauer & Mason, 1970; Ichikawa, Morioka & Sawada, 1971) Their rate of production and secretion varies during the oestrous cycle and it has been suggested that phase dependent changes in mood or behaviour during the sexual cycle can be caused by these reduced steroids (Holzbauer, 1971b). In addition it may be possible that they depress the activity of those hypothalamic neurons which stimulate the release of the luteinizing hormone releasing factor and may thus play a major role in a feed back system. Observations on their secretion during pregnancy and other factors which influence their production rate will be discussed.

References

HOLZBAUER, M. (1969). Pregnenolone and metabolites of progesterone in the ovary. *J. Physiol.*, **204**, 8-10P.

HOLZBAUER, M. (1971a). Ovarian secretion of steroids with central depressant actions. *J Physiol*, **215**, 16P.

HOLZBAUER, M. (1971b). *In vivo* production of steroids with central depressant actions by the ovary of the rat. *Br. J. Pharmac.*, **43**, 560-569.

HOLZBAUER, M. & MASON, P. (1970). Increase in the ovarian content of progesterone metabolites during late proestrus in the rat. *J Physiol.*, **210**, 128-130P.

ICHIKAWA, S., MORIOKA, H. & SAWADA, T. (1971). Identification of the neutral steroids in the ovarian venous plasma of LH-stimulated rats. *Endocrinology*, **88**, 372-383.

γ-Aminobutyric acid metabolism and the anticonvulsant action of ethanolamine-o-sulphate and di-n-propylacetate

G.M. ANLEZARK, R.W. HORTON*, B.S. MELDRUM, M.C.B. SAWAYA & J.D. STEPHENSON

Departments of Neurology and Pharmacology, Institute of Psychiatry, De Crespigny Park, London SE5 8AF

We have evaluated the anticonvulsant effects of ethanolamine-o-sulphate (EOS) and di-n-propyl acetate (DPA) in two test systems, audiogenic seizures in genetically susceptible mice (DBA/2, 21-25 days old) and picrotoxin-induced seizures in chicks (5-6 days old, Rhode Island Red) and correlated changes in behaviour and seizure response to changes in brain γ-aminobutyric acid (GABA) concentration and the activity of enzymes involved in GABA catabolism.

In mice EOS was administered intracerebroventricularly 24 h before test. At 7.5 mg/kg 50% of the animals and at 15 mg/kg 80% showed mild to moderate ataxia and were completely protected against the convulsant effect of auditory stimulation. The remainder showed no behavioural effects, and were not protected or showed partial protection against audiogenic stimulation. GABA transaminase (GABA-t) activity was inhibited 54% and 68% and cerebral GABA concentration increased 4 and 10 fold, after 7.5 and 15 mg/kg respectively. Succinic semialdehyde dehydrogenase (SSADH) activity was not altered.

In chicks, EOS (300 or 600 mg/kg given i.p. 48 h earlier) inhibited GABA-t activity by 54-59% and doubled brain GABA concentration but only raised the ED_{50} for picrotoxin seizures by 30-40% (not significant).

In mice, DPA was administered i.p. 45 min before test. Seizure responses were unaffected at 200 mg/kg, severely reduced at 400 mg/kg and completely absent at 600 mg/kg. Slight behavioural effects were seen only after 600 mg/kg. GABA concentration and GABA-t activity were unchanged after 200 or 400 mg/kg. GABA concentration was increased by 57% and GABA-t activity inhibited by 33% after 600 mg/kg. SSADH activity was unchanged.

In chicks, DPA (400 or 800 mg/kg i.p., 0.5-1 h before test) produced a 33% or 100% increase respectively in the ED_{50} for picrotoxin seizures. Brain GABA concentration was increased 16-30%, but GABA-t was inhibited only by 4-6%.

DPA is claimed to be a competitive inhibitor of GABA-t *in vitro* (Simler, Ciesielski, Maitre, Randrianarisoa & Mandel, 1973). Our results with mouse brain homogenates show that DPA is a poor *in vitro* inhibitor of GABA-t, but a potent competitive inhibitor of SSADH activity, confirming the findings of Harvey, Bradford & Davison (1975).

In these mouse and chick test systems it is difficult to relate the anticonvulsant properties of the two compounds to changes in the metabolism of GABA. The quantitatively smaller changes seen after DPA compared to EOS might be at physiologically more important sites. However there is clearly no correlation between anticonvulsant action and the accumulation of GABA in the brain.

We thank the Wellcome Trust and the National Fund for Research into Crippling Diseases for financial support and Dr L.J. Fowler (School of Pharmacy, University of London) for a generous gift of EOS.

References

HARVEY, P.K.P., BRADFORD, H.F. & DAVISON, A.N. (1975). The inhibitory effect of sodium n-dipropylacetate on the degradative enzymes of the GABA shunt. *Febs Letts,* **52,** 251-254.

SIMLER, S., CIESIELSKI, L., MAITRE, M. RANDRIANARISOA, H. & MANDEL, P. (1973). Effect of sodium n-dipropylacetate on audiogenic siezures and brain γ-aminobutyric acid level. *Biochem Pharmacol.,* **22,** 1701-1708.

DEMONSTRATIONS

D.1

The assessment of systemically and topically administered anti-inflammatory drugs using u.v. erythema production in the rat

A.J. LEWIS

Scientific Development Group, Organon Laboratories Limited, Newhouse, Lanarkshire ML1 5SH

The delaying action of u.v. induced erythema by non-steroidal but not steroidal anti-inflammatory agents has been demonstrated using the guinea-pig (Winder, Wax, Burr, Been & Rosière, 1958). This anti-erythemic activity correlates well with the anti-inflammatory action of non-steroidal agents. The present study describes a similar model in the rat which is susceptible to both non-steroidal and steroidal anti-inflammatory drugs (AID). Moreover, topical administration of both types of drug is also capable of suppressing the erythematous response.

Male Wistar rats (80-100 g) were shaved and depilated by Veet-O cream. Twenty-four hours later the rats were exposed to u.v. radiation provided by a Hanovia Model 10 Quartz Lamp fitted with a Kodak 18A glass filter transmitting light above 290 nm wavelength. Erythema, assessed subjectively by two observers, was visible 1 h after u.v. exposure for 90 seconds. The maximal response occurred by 4 h in areas exposed for 90 s and longer and was replaced by scab formation by 48 hours

Drugs were administered subcutaneously or orally in 5% mulgofen 1 h prior to u.v exposure. Erythema was then assessed at 2, 4 and 24 h after irradiation. For topical administration the drugs were dissolved in ethanol and administered to rats after u.v. exposure. This was achieved by the application of the drug solution (0.2 ml) into a small cylindrical reservoir which surrounded the irradiated skin. Control irradiated animals received an equal volume of ethanol. These areas were immediately covered with Sleek plastic adhesive to prevent drug ingestion and the erythema was assessed 4 h later after removal of this dressing.

Drug treatments were randomized for all routes of administration and the assessors were unaware of the treatment of individual groups.

Both steroidal and non-steroidal AID administered subcutaneously and orally, respectively, delayed the erythema in a dose-related fashion. However, systemically administered steroidal AID suppressed the erythema by less than 50% at doses capable of inhibiting kaolin-induced paw oedemas by greater than 50%. Oral ID_{50} values (mg/kg in parenthesis) have been obtained for several non-steroidal AID including aspirin (148), ibuprofen (22), indomethacin (1.3) and phenylbutazone (17).

The topically administered drugs also produced dose-related suppression of the erythema and ID_{50} values are described in Table 1. A new non-halogenated steroidal agent, Org 6216 (11β-hydroxy-16α,17α,21-trimethyl-pregna-1,4-diene-3,20-dione), possessed very potent topical activity, similar to that shown by indomethacin.

In conclusion, a simple model is described that may be useful for the systemic and topical screening of compounds of potential use as anti-inflammatory drugs.

Table 1 Anti-erythema effects of topically administered AID

Drug	*ID_{50}(mg/rat)*
Org 6216	1 0
Indomethacin	1.3
Dexamethasone	4.8
Phenylbutazone	5.6
Betamethasone-17-valerate	7.8
Hydrocortisone	9.0
Aspirin	9.2
Flufenamic acid	10.9

Drugs administered in 0.2 ml ethanol immediately after u.v. exposure (90 s). Erythema was assessed 4 h after irradiation.

Reference

WINDER, C.V., WAX, J., BURR, V., BEEN, M. & ROSIÈRE, C.E. (1958). A study of pharmacological influences on ultraviolet erythema in guinea pigs. *Arch. int. Pharmacodyn.*, **116**, 261-292.

A rapid in vivo test for dependence potential of analgesic drugs

N.J. CUTHBERT, L.C. DINNEEN, D.L. FRANCIS & C. SCHNEIDER

Research Department, Miles Laboratories Limited, Stoke Poges, Slough

Collier, Francis, Henderson & Schneider (1974) observed that heroin (1 mg/kg s.c) reduced the incidence of the quasi-morphine abstinence syndrome (QMAS) produced by giving a single dose of theophylline orally to naive rats. The effect of heroin was removed by the simultaneous administration of naloxone (0.2 mg/kg). More recently, Collier & Francis have shown that whereas levorphanol reduced the quasi-morphine abstinence effect induced by another methylxanthine, 3-isobutyl-1-methylxanthine (IBMX) its stereoisomer dextrorphan had no effect. These results suggested therefore that suppression by narcotic drugs of the QMAS could be used as a rapid *in vivo* method for assessing dependence potential, in a similar way to the conventional single dose suppression test, an example of which was described by Collier & Schneider (1972). Of particular interest in the present series of experiments were the actions of several orally effective drugs commonly used to relieve pain in man, that had differing dependence potential but similar analgesic potency.

The dose-response line for pentazocine suppressing QMAS was shallower that that of codeine, *d*-propoxyphene or pethidine. Rats given an opiate with IBMX and challenged 30 min later by naloxone (0.3 mg/kg) showed a significant dose-related increase in withdrawal jumping, compared with rats given an opiate or IBMX alone. Thus, the physical dependence potential of an opiate may be due to its reducing indirectly, cyclic AMP phosphodiesterase activity in morphine-sensitive neurones.

References

COLLIER, H.O.J., FRANCIS, D.L., HENDERSON, G. & SCHNEIDER, C. (1974) Quasi-morphine abstinence syndrome. *Nature Lond.*, **249**, 471-473.

COLLIER, H.O.J. & FRANCIS, D.L (1975). Stereospecific suppression by opiates of the quasi-morphine abstinence syndrome elicited by 3-isobutyl-1-methylxanthine (IBMX). *Br. J. Pharmac* (in press).

COLLIER, H.O.J. & SCHNEIDER, C. (1972) Some agonist and antagonist properties of morphine antagonists in rodents. In: *"Agonist and Antagonist Actions of Narcotic Analgesic Drugs"*. Edited in Kosterlitz, H.W., Collier, H.O.J. & Villarreal, J.E., pp. 128-135.

Sympathetic nerve recording in the conscious rabbit

G. HAEUSLER & P.J. LEWIS

Experimental Medicine Department, F. Hoffmann La Roche, Basle, Switzerland and Clinical Pharmacology Unit, Queen Charlotte's Hospital, London

The recording of electrical activity from peripheral sympathetic nerves is a useful technique for the evaluation of centrally acting hypotensive drugs. Records of pre- or postganglionic sympathetic activity can be obtained by placing the splanchnic or renal nerve on bipolar electrodes and covering the preparation with mineral oil. However, such recordings can only be made in acute experiments in anaesthetized or curarized animals and such conditions are not ideal for studying centrally acting drugs whose effects may be modified by anaesthesia. Schmitt, Schmitt & Fenard (1974) described implantation of electrodes in the dog and we have adopted this technique for chronic recordings in the rabbit.

Rabbits of 2-3 kg were anaesthetized with halothane and the abdomen opened transversely along the right costal margin. The right greater splanchnic nerve was located anterior to the crus of the diaphragm and a 1.5 cm length cleared of perinephric fat. The nerve was then transfixed by the two prongs of a bipolar electrode. The electrode tips consisted of 0.3 mm stainless steel wire which had been sharpened electrolytically and mounted in a block of epoxy resin 3 mm apart. Flexible wires from the electrode were led out onto the skin of the back. During implantation electrical activity was monitored via a low level D.C. amplifier and oscilloscope (Tektronix USA) and when a satisfactory signal was obtained the electrode was fixed in position by the application of acrylic dental cement. Several grams of this material were deposited over the nerve and electrode. The abdomen was then closed and an indifferent electrode stitched beneath the skin.

Recordings have been made in the conscious animal up to 30 days after implantation. The pattern of electrical activity is characteristically phasic, bursts of activity being synchronized with respiration. The discharge amplitude varies between 20 and 100 μv. In 20 consecutive preparations, 12 animals had satisfactory recordings made on the day after implantation. If the signal was satisfactory on the first day it remained so thereafter although animals were lost when wires were bitten through. Integrated activity was recorded in 7 animals for 20 min on consecutive days following implantation. Activity was greatest on day 1 and stabilized on days 4 and 5 at about 50% of the initial value. Reduction in arterial pressure with intravenous sodium nitroprusside or haemorrhage produced reflex rises in integrated activity. Anaesthesia with sodium pentobarbitone 30 mg/kg i.v. significantly reduced splanchnic nerve activity to 58 ± 8% (s.e mean, $n = 5$) of control 5 min after injection.

Rabbits are particularly suitable animals for these experiments because they remain quiet when placed in individual boxes and thus stable recordings of sympathetic activity can be made for several hours. We have used the technique for the evaluation of central and peripheral effects of beta blocking drugs (Lewis & Haeusler, 1975) and it has potential use in monitoring sympathetic nervous activity in chronic experiments.

References

LEWIS, P.J. & HAEUSLER, G. (1975). Reduction in sympathetic nervous activity as a mechanism of hypotensive effect of propranolol. *Nature Lond.*, 256, 440.

SCHMITT, H., SCHMITT, H. & FENARD, S. (1974). A technique for recording sympathetic nerve activity in unanaesthetized dogs. *Neuropharmacology*, 13, 347-351.

In vitro methods of assessing the physiological activation of macrophages in vivo

N.J. BRADLEY & L.W. POULTER (introduced by G.P. LEWIS)

Department of Pathology, Royal College of Surgeons of England, London WC2A 3PN

The activation of macrophages, as assessed by a spreading and vacuolation of the cytoplasm and increases in respiratory and lysosomal enzyme activity, has been shown to occur during infection and various chronic inflammatory reactions. There is much circumstantial evidence that this activation *in vivo* is mediated by lymphokine.

In vitro, macrophages can be activated by prolonged contact with lymphokine (Nathan, Karnovsky & David, 1971; Nathan, Remold & David, 1973; Nath, Poulter & Turk, 1973). This activation only occurs however after the initial effects of lymphokine (which result in migration inhibition) have waned. The initial changes to the physiology of the macrophage appear to be an alteration in the utilization of the hydrogen liberated by the hexose monophosphate shunt, which results in reduced biosynthetic potential and a decrease in cellular permeability. These changes occur concurently with an inhibition of migrating ability as seen in the capillary tube assay. After 48-72 h of contact with lymphokine, these initial effects are reversed and increases in hexose monophosphate shunt activity and biosynthesis are seen as well as morphological changes, which result in the macrophages appearing similar to cells activated *in vivo*.

Because of this biphasic effect, it was felt that any contact with lymphokine *in vivo* would result in an altered response to subsequent lymphokine contact *in vitro*. This hypothesis has been tested by removing macrophages from animals at various times during a chronic protozoal infection (Leishmaniasis) and also after reinfecting immune animals and then recording the subsequent response of these cells to the lymphokine *in vitro*.

This was done by assaying the ability of these macrophages to respond in direct and indirect migration inhibition assays, and also by examining the effect of lymphokine contact on the activity of the hexose monophosphate shunt as detected by cytochemical tests for glucose-6-phosphate dehydrogenase activity.

The results of these studies indicate that changes in the status of macrophages during infection and following attempted reinfection can be detected by these methods, and these changes are consistent with the hypothesis that macrophage activation *in vivo* is mediated by contact with lymphokine.

References

NATHAN, C.F., KARNOVSKY, M.L. & DAVID, J.R. (1971). Alterations of macrophage functions by mediators from lymphocytes. *J. exp. Med.*, 133, 1356-1376.

NATHAN, C.F., REMOLD, H.G. & DAVID, J.R. (1973). Characterization of a lymphocyte factor which alters macrophage functions. *J. exp. Med.*, 137, 275-290.

NATH, INDIRA, POULTER, L W. & TURK, J.L. (1973). Effect of lymphocyte mediators on macrophages *in vitro* A correlation of morphological and cytochemical changes. *Clin. exp. Immunol.*, 13, 455-466.

The effect of apomorphine on oral behaviour in piglets

J.P. FRY, D.F. SHARMAN & D.B. STEPHENS

Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge CB2 4AT

The new-born mammal has both nutritive and non-nutritive sucking requirements (Levy, 1934). When non-nutritive sucking is prevented, abnormal oral behaviour which can resemble the effect of apomorphine may be induced. The two types of sucking behaviour can be distinguished using equipment described by Stephens (1975). This consists of a wooden box lined with rubber sheeting. A rubber teat is inserted through a hole in one wall. The teat is connected to two microswitches which record teat movements and milk flow through the teat is also monitored. One-day-old piglets can be trained to feed from the teat in three days at which time they develop a regular pattern of teat activity. This consists of an ingestive phase lasting approximately 15 min during which the piglets drink the milk provided. Then follows a period of sporadic non-nutritive teat activity when the piglets nuzzle at or around the teat without drinking. Low doses of apomorphine (0.1-0.2 mg/kg s.c.) injected during the latter phase greatly prolong and intensify the non-nutritive teat activity. This method is useful for quantifying behavioural effects of apomorphine. The responses of other farm animals to apomorphine and other drugs thought to affect central dopaminergic neuron systems will also be shown and compared with abnormal oral behaviour seen under intensive husbandry conditions.

J.P.F. was supported by an A.R.C. Research Studentship.

References

LEVY, D.M. (1934). Experiments on the sucking reflex and social behaviour of dogs. *Am. J. Orthopsychiat.*, 4, 203-224.

STEPHENS, D.B. (1975). Effects of gastric loading on the sucking response and voluntary milk intake in neonatal piglets. *J. comp. physiol. Psychol.*, 88, 796-805.

Measurement of vascular changes in acute inflammatory responses

HELEN E. FARRINGTON & D.A.A. OWEN

Department of Pharmacology, The Research Institute, Smith Kline and French Laboratories Ltd., Welwyn Garden City, Hertfordshire

Acute inflammatory responses are associated with increases in blood flow and accumulation of plasma proteins within the inflammatory lesion. We have modified existing techniques to measure the vascular changes caused by acute inflammatory stimuli in rat hindpaws. Experiments have been made in male rats, body weight 160-200 g, anaesthetized with urethane (1.25 g/kg i.p.). The inflammatory stimulus was applied to one paw and the other paw served as a control. Paw blood content was measured using [^{51}Cr]-labelled rat red blood cells (approximately 1 μCi) and accumulation of albumin in the tissue using [^{125}I]-labelled human serum albumin (approximately 250 nCi), each injected intravenously 5 min before the inflammatory stimulus. Paw blood and albumin content were expressed as volume in terms of venous blood. Blood flow was measured using [^{85}Sr]-labelled microspheres, 25 μ diameter (3M Company). The microspheres were injected into the left ventricle of the heart via a catheter in the right carotid artery. Blood flow to each hindpaw was expressed as a % of cardiac output and flow to the injured paw was also expressed as a % of the flow to the control paw. After injection of the microspheres the rats were killed and both paws removed and placed in vials to permit differential γ-counting of the ^{85}Sr, ^{51}Cr and ^{125}I content of the paws using a Packard autogamma scintillation spectrometer.

Table 1 shows the results from a typical group of experiments in which the inflammatory

Table 1 Local vascular changes caused by immersion of rat hindpaws in water at 60°C for 30 s, $n = 5$

	Oedema	*Blood flow*		*Blood volume*		*Albumin accumulation*	
	Injured paw weight % of control paw	*% cardiac output*	*% of flow in control paw*	*ml*	*% of content in control paw*	*ml*	*% of content in control paw*
Control paw	100	0.29± 0.06	100	0.05± 0.01	100	0.06± 0.01	100
Injured paw	132.2± 4.1	3.71± 0.56	1279	0.13± 0.01	260	0.74± 0.11	1233

stimulus was immersion of the paw in water at 60°C for 30 seconds. The changes measured 15 min after the burn indicate a large increase in blood flow to the injured paw with marked accumulation of albumin in the tissue and a small increase in blood content.

The use of a mass spectrometer for the analysis and measurement of trace concentrations of anaesthetic vapours

J.A. BUSHMAN, D.H. ENDERBY, K. T. FOWLER & J.P. PAYNE

Research Department of Anaesthetics, Royal College of Surgeons, London WC2A 3PN

The possibility of untoward effects resulting from the long term exposure to low concentrations of certain anaesthetic vapours has recently caused concern amongst some anaesthetists. Various methods have been devised to minimize the concentration of these vapours in the operating theatre and in order to judge their effectiveness it has been necessary to devise a method of accurately measuring the concentration of the vapour remaining in the operating room. Since other vapours may be present in relatively high concentrations it is important to ensure the method used is specific for the particular vapour being studied. It is also important to have a method of providing standard concentrations of the gas in the parts per million range.

An ideal instrument for such an investigation is a mass spectrometer since it is capable of measuring very low concentrations and can be specific for any particular vapour under investigation. The instruments that will be demonstrated are quadrupole mass spectrometers capable of measuring ions of up to 200 at. mass units in concentrations as low as one part per million.

A method of producing calibration vapours in the parts per million range by continuous serial dilution has been developed. The problems associated with stabilizing high flow rates through rotameters were overcome by using a fluidic device which ensured a high stability of flow rate into the dilution circuit.

The technique is at present being used to study the rate of loss of vapours from anaesthetic tubing both during use and during periods when, though not in use, vapours absorbed by the tubing may pollute the surrounding atmosphere.

Assessment of neuromuscular blockade in man by use of tetanic and single twitch contractions of the adductor pollicis muscle

D.H. ENDERBY, R. HUGHES, J.P. PAYNE & R. WORSLEY

Research Department of Anaesthetics, Royal College of Surgeons of England, London WC2 and St. Peter's Hospital, London WC2

Studies were carried out after informed consent had been obtained in patients about to undergo urological surgery. Anaesthesia was induced with thiopentone and endotracheal intubation was carried out after spraying the larynx with 4% lignocaine. Anaesthesia was maintained with nitrous oxide and oxygen supplemented by pentazocine (60-120 mg) and additional doses of thiopentone.

Simultaneous measurements of tetanic and single twitch contractions of the adductor pollicis muscles were measured by Statham force transducers (Figure 1) incorporated in hand grips. Fine needle electrodes were inserted subcutaneously at the wrists and the ulnar nerves were stimulated every 12 s with rectangular pulses of 0.2 ms duration and supramaximal voltage from an isolated stimulator. One ulnar nerve received tetanic pulses at 50 Hz for 1 s, the other single shocks. The temperature of each hand was monitored with a surface probe, any differences were within 1° C.

The radial artery was cannulated at the wrist exposed to single shocks. Arterial blood samples were taken for analysis of blood gases which were maintained within normal limits by assisted or controlled ventilation.

The responses of the muscles were recorded at a slow chart speed of 5 mm/min on a Brush-Clevite recorder and at a fast speed of 50 mm/s on a Mingograf recorder, together with blood pressure and the electrocardiogram.

Tetanic-tension ratios and tetanic transmissions were determined by an Elliott 903 digital computer from on-line real-time data. The tetanic-tension ratio, which is defined as the percentage magnitude of the tetanic contractions at the end of the 1s tetanus compared with the initial magnitude, reflects the degree of fade produced during neuromuscular block. Tetanic transmission was determined as the percentage of the initial peak tetanic height compared with the control-peak height before drug treatment.

These facilities will be demonstrated in the off-line mode using tape recordings to show the effects of known neuromuscular blocking agents.

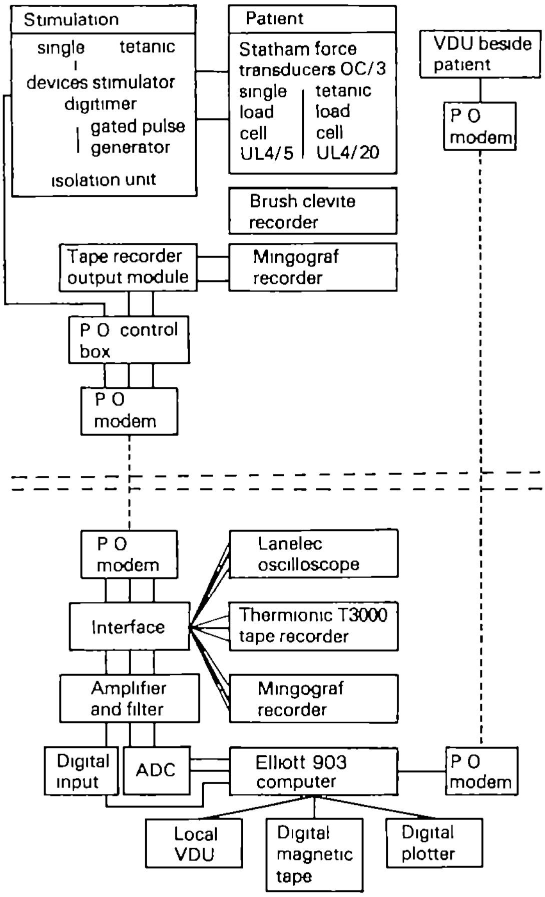


Figure 1 Schematic diagram of the recording equipment and facilities for computer analysis.

Measurement of changes in the microvasculature

R.T. HUTCHINGS, G.P. LEWIS, Z.T. SABIKOWSKI & J. WESTWICK

Departments of Anatomy and Pharmacology, Royal College of Surgeons, London WC2A 3PN

This inexpensive instrument was designed to record *in vivo* any changes in a microvessel which would result in a change of optical density, e.g. alteration of vessel diameter, white thrombus formation, white cell rolling.

The apparatus consists of an optical adaptor, photocell and amplifier (Figure 1). It can be used in normal daylight with any research microscope having a phototube and a magnification factor of up to 100-350 x and a tungsten light source.

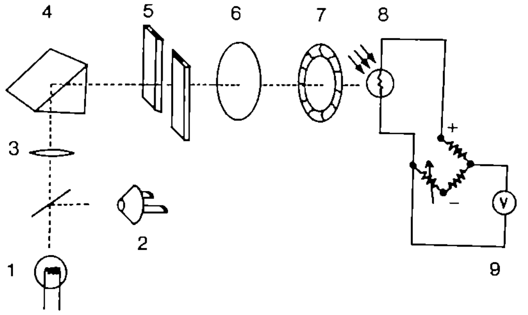


Figure 1

1. Tungsten light source
2. Binocular viewing tube
3. Phototube lens
4. Prism
5. Adjustable knife edge
6. Ground glass screen
7. Adjustable iris diaphragm
8. Photocell
9. Pen recorder

The optical adaptor consists of a prism attached above the projecting eyepiece of a standard Leitz camera tube. The beam is turned through 90° and projected onto a small ground glass viewing screen. A CdS photocell (cadmium sulpho selenide photoconductive cell – Mullard RPY 33) is placed in front of the screen after the experiment has been set up. To reduce glare and increase contrast, an optical knife edge is placed behind the screen and an iris diaphragm in front. Manipulation of the masks enables a square or rectangle to be formed. The photocell is recessed into a black perspex disc and when placed into position excludes all extraneous light. The experiment can be monitored using the binocular viewing head of the microscope The pen recorder can be calibrated by aligning the vessel with the graticules in the eye-piece and on the ground glass screen.

The high sensitivity CdS photoconductive cell used is connected to a Wheatstone bridge circuit. The circuit is adjusted to a zero balance with a multi-turn potentiometer and balance meter. A local variation of light intensity results in a conductivity change in the photocell which unbalances the bridge circuit and produces an output voltage in excess of 50 mV at 2.5 kΩ impedance. The voltage is then filtered out with an RC filter and recorded on a continuous potentiometric pen recorder.

This work was partly supported by a grant from Ciba/Geigy Ltd.

A simple perfusion system for the study of histamine release from rat peritoneal mast cells

U. NIEDERHAUSER & B.J.R. WHITTLE

Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons of England, Lincoln's Inn Fields, London WC2A 3PN

Rat mast cells are extensively used to investigate the processes involved with the release of inflammatory mediators from cells. In the present study, a simple and rapid perfusion technique has been developed for the determination of histamine release from rat peritoneal mast cells.

Mast cells were obtained by gentle lavage of the rat peritoneal cavity with 12 ml of a modified buffer solution at pH 7 (Uvnäs & Thon, 1961) containing bovine serum albumin (0.1% w/v). The solution was withdrawn from the cavity and stored on ice until required. Aliquots (1-4 ml) were passed through a nylon membrane (duralon, 1 μm pore size) encased in a millipore filter-holder (Swinnex-25). A heating jacket was used to control the temperature of the filter-holder. In 20 experiments, the histamine content of the perfusate, before and after filtration, was comparable (within 5.8 ± 2.9%, mean ± s.e. mean), suggesting that this procedure did not disrupt the cells. Further, comparison of the total histamine content of the perfusate (using 0.4 N perchloric acid to disrupt any cells present) indicated that over 90% of the mast cells were trapped on the membrane, a finding confirmed by microscopy. The cells were then perfused (2 ml/min) with albumin-free buffer solution, and the perfusate collected at timed intervals. The histamine content of the perfusate was determined by alkaline condensation with O-phthalaldehyde followed by acidification and measurement of the fluorescence (excitation 350 nm, emission 440 nm; Shore, Burkhalter & Cohn, 1959) or by bioassay on the superfused guinea-pig ileum. In further experiments, the perfusate was led directly to a mixing coil and to a flow-cell spectrofluorimeter which enabled the chemical histamine assay to be carried out automatically, with the fluorescence intensity being displayed on a chart recorder.

The cells appeared to retain their functional integrity since the low resting histamine output could readily be increased up to 200-fold by chemical or immunological challenge. In 12 experiments, administration of the polymer 48/80 (0.1 ml volume) into the perfusion medium gave a reproducible and dose-dependent release of histamine from the cells. The threshold (0.05-0.1 μg) and maximal (1-2 μg) doses of 48/80 were similar to those observed with incubated mast cells. With initial high doses of 48/80 (0.5-2 μg), subsequent administration in the same experiment gave a very diminished histamine release, presumably due to depletion of the stored mast cell histamine. However, with lower doses of 48/80 (0.05-0.2 μg), several comparable responses could be obtained.

This method, which can readily be adapted to study other mediators such as 5-hydroxytryptamine, and other cell types, should give a better understanding of the kinetics of mediator release from specific inflammatory cells and provide a simple technique for the rapid screening of drugs which alter such release.

This work was supported by a grant from the M.R.C. to Professor G.P. Lewis.

References

SHORE, P.A., BURKHALTER, A. & COHN, V.H. (1959). A method for the fluorometric assay of histamine in tissues. *J. Pharmac. exp Ther.*, 127, 182-186.

UVNAS, B. & THON, I-L. (1961). Evidence for enzymatic histamine release from isolated rat mast cells. *Expl. Cell Res.*, 23, 45-57.

The inhibitory transmission to the internal anal sphincter

A.O. ADEBANJO, N. AMBACHE & J. VERNEY

Medical Research Council, Department of Physiology, Royal College of Surgeons of England, London WC2A 3PN

Inhibition of this sphincter by the sacral nerves is atropine-resistant (Langley & Anderson, 1895a; Garrett & Howard, 1975). Our results show that this sacral inhibitory post-ganglionic transmission resembles that present in the retractor penis (Luduena & Grigas, 1966, 1972; Ambache, Killick & Zar, 1975). It is likely that the other atropine-resistant sacral inhibitory effects described by Langley & Anderson (1895b) are similarly mediated.

Procedure: The anal canal is excised by a wide perineal incision. The anus is slit and a 1-2 cm width of mucosa, adjoining the skin, is dissected off the underlying sphincters. A plane of separation is found between the pink-brown external sphincter (skeletal) and the white smooth muscle band of the internal sphincter. Strips from > 50 rabbit, pig, dog, cat and guinea-pig sphincters were suspended in Krebs-Henseleit (35° C) and stimulated transmurally (1-20) pulses at 1 min intervals; supramaximal voltage, 0.2 ms 10 Hz).

Results: Transmural stimulation elicited repeatable inhibitions unaffected by pentolinium (93 μM) but abolished by tetrodotoxin (0.63 μM). Pure relaxations were obtained without 'rebound' (e.g. Figure 1) in most preparations; only a few had an additional (adrenergic) motor component which disappeared after guanethidine, 10 μM, or phentolamine, 5.3 μM.

That acetylcholine is not the inhibitory parasympathetic transmitter was shown by: (a) its opposite, contractile effect, except once (cat sphincter; cf. monkey, Rayner, 1971) when, in high doses (277 μM), acetylcholine, like nicotine, produced a pentolinium-susceptible inhibition. (b) Persistence of these neurogenic inhibitions in atropine (0.029-29 μM); and (c) failure of physostigmine (7.7 μM) to potentiate the atropine-resistant inhibitions.

The inhibitory transmission is not adrenergic or tryptaminergic, persisting after propranolol

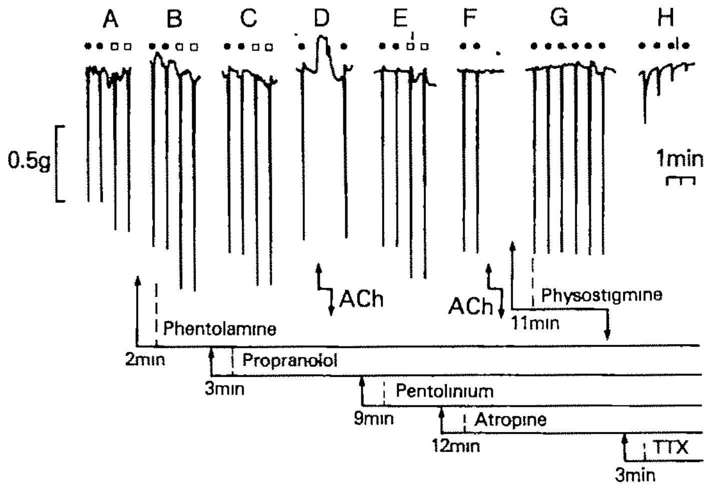


Figure 1 Rabbit sphincter ani internus. neurogenic inhibition is not cholinergic or adrenergic. The inhibitory responses, elicited at 1 min intervals by 10 (●) or 20 (□) pulses, persist (B-G) in phentolamine (5.3 μM), propranolol (6.8 μM), pentolinium (93 μM) and atropine (29 nM) but vanish, at H, in tetrodotoxin (TTX, 0.63 μM) They are not mimicked by 28 μM acetylcholine (ACh, 28 μM), which is motor before atropine (cf. D and F); and are not potentiated by physostigmine (G, 7 7 μM). The preparation did not respond to ATP (18 μM; not shown).

(6.8 μM) or butoxamine (6.6 μM) ± phentolamine (5.3 μM) or phenoxybenzamine (5.8 μM); and after guanethidine (10 μM), bretylium (24 μM) or LSD (125 nM).

Transmission by ATP, histamine or prostaglandins (E and F), was excluded.

A.O.A. is supported by the University of Ife, Nigeria.

References

AMBACHE, N., KILLICK, S.W. & ZAR, M. ABOO. (1975). Extraction from ox retractor penis of an inhibitory substance which mimics its atropine-resistant neurogenic relaxation. *Br. J. Pharmac.*, 54, 409-410.

GARRETT, J.R. & HOWARD, E.R. (1975). Neural control of the internal anal sphincter of cats after chemical sympathectomy with 6-hydroxydopamine. *J. Physiol., Lond.*, 247, 25-27P

LANGLEY, J.N. & ANDERSON, H.K. (1895a). On the innervation of the pelvic and adjoining viscera. Pt. I. The lower portion of the intestine. *J. Physiol., Lond.*, 18, 67-105

LANGLEY, J.N. & ANDERSON, H.K. (1895b). The innervation of the pelvic and adjoining viscera. Pt. III. The external generative organs. *J. Physiol., Lond.*, 19, 85-121.

LUDUENA, F.P. & GRIGAS, E.O. (1966). Pharmacological study of autonomic innervation of dog retractor penis. *Am. J. Physiol.*, 210, 435-444.

LUDUENA, F.P. & GRIGAS, E.O (1972). Effect of some biological substances on the dog retractor penis *in vitro*. *Arch. int. Pharmacodyn.*, 196, 269-274.

RAYNER, V. (1971). Observations on the functional internal anal sphincter of the vervet monkey. *J. Physiol, Lond*, 213, 27-28P.

Effect of a purified cotton dust extract on human lung in culture

J.H. EDWARDS, ELIZABETH EVANS, P.H. EVANS, P.J. NICHOLLS & K.T. RAJAN

M.R.C. Pneumoconiosis Unit, Llandough Hospital, S. Glam., Welsh School of Pharmacy, UWIST, and University Hospital of Wales, Cardiff

The lung function changes observed in byssinotic cotton workers following the inhalation of cotton dust have been attributed to the presence of a water-soluble pharmacologically active agent in the dust (McKerrow, McDermott, Gilson & Schilling, 1958). Indeed, Davenport & Paton (1962) described the presence of several pharmacological activities in such dust extracts. There is accumulating evidence that the acute lung function changes may be associated with the presence of histamine releasing activity in the dust (Bouhuys, 1974). Recently, the major histamine-releasing component of cardroom cotton dust has been isolated by thin layer chromatography and tentatively identified as an aminopolysaccharide-protein complex (Evans & Nicholls, 1974). As part of an examination of the pharmacological properties of this substance, its action upon human lung in culture has been investigated.

Lung lobules from 8-12 week-old human embryos were maintained in organ culture in a synthetic medium (BGJ5, Biggers, Gwatkin & Heyner, 1961) containing 15% w/v foetal calf serum and ascorbic acid, 150 μg/ml (Reynolds. 1972). The organ culture technique of Trowell (1954) as modified by Dingle, Fell & Lucy (1966) was employed. The medium was changed on alternate days and cultures were maintained for 8 days at 37° C in an atmosphere of O_2 (50%) and CO_2 (5%). Under these conditions, the cultures remained viable for 8 days and there was growth and differentiation as seen by light microscopy. Some explants were maintained in media containing the cotton dust aminopolysaccharide-protein complex.

Of 12 explants examined there were no abnormalities demonstrated histologically when incubation was in the control medium and in media containing the dust fraction at a concentration of 200 μg/ml. However, explants exposed to this material in a concentration of 400 μg/ml showed the following features: (a) tissue necrosis, most marked in the broncial epithelium and (b) in areas where the bronchial epithelium was intact, marked exudate in the lumen consisting of PAS-staining material and numerous necrotic cells. There was also a marked increase in the amount of the lysosomal enzyme β-glucuronidase released from lung into the medium by this higher concentration of the cotton dust fraction at 2, 4, 6 and 8 days in culture. This was additional to a low level of β-glucuronidase activity exhibited by this concentration of the aminopolysaccharide-protein fraction alone.

E.E. is in receipt of a Victor Reed Scholarship from the Pharmaceutical Society of Great Britain.

References

BIGGERS, J.D., GWATKIN, R.B.L. & HEYNER, S. (1961). Growth of embryonic avian and mammalian tibiae on a relatively simple chemically defined medium. *Exp. Cell. Res.*, **25**, 41-58.

BOUHUYS, A. (1974). *Breathing Physiology, Environment and Lung Disease*, pp. 422-431. New York and London: Grune and Stratton.

DAVENPORT, A. & PATON, W.D.M. (1962). The pharmacological activity of extracts of cotton dust. *Br. J. industr. Med.*, **19**, 19-32.

DINGLE, J.T., FELL, H.B & LUCY, J.A. (1966) Synthesis of connective tissue components. The effect of retinol and hydrocortisone on cultured limb-bone rudiments. *Biochem. J.*, **98**, 173-181.

EVANS, E. & NICHOLLS, P.J. (1974). Preliminary characterization of the histamine releasing activity of cotton dust. *J. Pharm. Pharmac.*, **26**, 115-116P.

McKERROW, C B., McDERMOTT, M., GILSON, J.C. & SCHILLING, R.S.F. (1958) Respiratory function during the day in cotton workers: a study in byssinosis. *Br J. industr. Med.*, **15**, 75-83.

REYNOLDS, J.J. (1972). Skeletal tissue in culture. In *The Biochemistry and Physiology of Bone.* Vol. 1. Second edn. New York: Academic Press.

TROWELL, O.A. (1954). A modified technique for organ culture *in vitro. Exp. Cell. Res.*, **6**, 246-248.

Reduction of locomotor activity in mice by dopamine-β-hydroxylase inhibitors – evidence against the involvement of non-specific irritation

A.C. DOLPHIN, P.N.C. ELLIOTT & P. JENNER

Department of Neurology, Institute of Psychiatry and King's College Hospital Medical School, Denmark Hill, London SE5 8AF

Dopamine-β-hydroxylase (DBH) inhibitors are widely used as research tools to distinguish between dopaminergic and noradrenergic mechanisms However, it has been suggested (Moore, 1969; Thornburg & Moore, 1973) that the effects of DBH inhibitors on reducing spontaneous motor activity are not causally related to the depletion of brain noradrenaline which they produce, but to the stress provoked by local irritation which is reflected in the rise in blood glucose and plasma corticosterone which occurs after their intraperitoneal administration (Thornburg & Moore, 1971).

We have tested this hypothesis by comparing two DBH inhibitors with two known irritants, for their ability to produce irritation and their effect on motor activity. The two DBH inhibitors used were 1,1-dimethyl-3-phenyl-2-thiourea (U10,157) and bis(4-methyl - 1 - homopiperazinylthiocarbonyl)-disulphide (FLA-63) in doses which are known to inhibit cerebral DBH. The two irritants used were colloidal carrageenan and kaolin. Their effects were compared on the induction of mouse paw oedema after subplantar administration, and in stimulation of peritoneal exudation after intraperitoneal administration as measured by leakage of intravenous Evans' Blue dye into the peritoneal cavity. Their effects were also compared on apomorphine and L-DOPA-induced motor activity. The results show that although FLA-63 and carrageenan in the concentrations used have a similar ability to produce inflammation and intraperitoneal irritation, carrageenan has no effect on motor activity produced by L-DOPA; neither the irritants nor the DBH inhibitors affect apomorphine-induced locomotor activity.

We conclude that although the DBH inhibitors tested are irritants, they are producing their effect on motor activity by a more specific direct mechanism which we believe to be related to depletion of cerebral noradrenaline.

References

MOORE, K.E. (1969). Effects of disulfuran and diethyl-dithiocarbamate on spontaneous locomotor activity and brain catecholamine levels in mice. *Biochem. Pharmacol.*, 18, 1627-1634.

THORNBURG, J.E. & MOORE, K.E. (1971). Stress-related effects of various inhibitors of catecholamine synthesis in the mouse. *Arch. Int. Pharmacodyn. Thér.*, 194, 158-167.

THORNBURG, J.E. & MOORE, K.E. (1973). The relative importance of dopaminergic and noradrenergic neuronal systems for the stimulation of locomotor activity induced by amphetamine and other drugs. *Neuropharmacol.*, 12, 853-866.

A pithed rabbit preparation for stimulation of different segments of the autonomic outflow

J.C. McGRATH & J.E. MacKENZIE

Department of Anaesthesia, University of Glasgow

Gillespie, MacLaren & Pollock (1970) described a method of stimulating different segments of the autonomic outflow from the spinal cord to various organs in the pithed cat and rat. We have modified this method for use in the rabbit. Since the geometry of the rabbit skull does not permit pithing via the orbit as in the cat and rat, this has been accomplished through a trephine-hole.

New Zealand white rabbits (3.0-3.5 kg) were anaesthetized with halothane (3%) and were decerebrated following placement of cannulae in the trachea, both carotid arteries (left, for arterial and left ventricular pressure; right, for thermistor for cardiac output), and a jugular vein (for administration of drugs). Halothane was then discontinued, gallamine (1 mg/kg i.v.) was given and mechanical ventilation with 100% O_2 (Harvard Respirator) was adjusted to give an end-tidal CO_2 of 3-4% (Beckman Medical Gas Analyser LB-2). Pithing was carried out using a teflon covered stainless steel rod identical to that used for the cat by Gillespie *et al.* (1970). The resulting preparation had a resting arterial pressure of 48 ± 3 mmHg systolic and 26 ± ~~2 mmHg~~ diastolic, and heart rate of 230 ± 4 min^{-1} ($n = 9$), which together with blood gas values remained stable over a period of at least 6 hours. As in the previous study in the rat and cat (Gillespie *et al.*, 1970), reproducible cardiovascular responses could be obtained to stimulation of the autonomic outflow (via the tip of the movable pithing rod) or to injected drugs.

In each animal, left ventricular pressure (and hence d Pv/dt) was also monitored via a cannula placed within that in the carotid artery. Cardiac output was measured by thermal dilution, enabling calculation of peripheral resistance. This preparation can assist in identifying the peripheral cardiovascular effects of drugs.

J.C.McG. received a Wellcome Trust Interdisciplinary Fellowship, and J.E.M. an M.R.C. Fellowship.

Reference

GILLESPIE, J.S., MacLAREN, A. & POLLOCK, D. (1970). A method of stimulating different segments of the autonomic outflow from the spinal column to various organs in the pithed cat and rat *Br. J. Pharmac.*, 40, 257-267.

The episcope for large screen projection of tracings on opaque media during their recording for demonstrations

J.B.E. BAKER

Department of Pharmacology, Charing Cross Hospital Medical School, London W6

Measurement of inflammatory exudation and local blood flow changes in rabbit skin, using [^{131}I]-albumin and ^{133}Xe

M.J. PECK & T.J. WILLIAMS (introduced by G.P. LEWIS)

Department of Pharmacology, Institute of Basic Medical Sciences, Royal College of Surgeons of England, London WC2A 3PN

Measurement of blood flow in rabbit skin homografts and autografts using a ^{133}Xe-clearance technique

G.P. LEWIS, M.J. PECK, T.J. WILLIAMS & BEVERLEY A. YOUNG

Department of Pharmacology, Royal College of Surgeons, London WC2A 3PN

Prostaglandin production by rat polymorphonuclear leucocytes

ELAINE McCALL & L.J.F. YOULTEN

Department of Pharmacology, Royal College of Surgeons, London WC2A 3PN

INSTRUCTIONS TO AUTHORS

Papers will be considered for publication on all aspects of drug action, including chemotherapy. A recent issue of the Journal is a good guide to style.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh, EH8 9JZ, from whom copies of the full Instructions to Authors, published in *Br. J. Pharmac*, **50**, 3-23, may also be obtained. Specific points to note are:

1. **Manuscript** should be typed in double spacing on one side of paper not larger than A4 (206 x 294 mm). There should be a separate title page giving the names and addresses of the authors **in alphabetical order.** A short title of not more than 50 letters should also be suggested.

2. **Statement** Papers are accepted only if accompanied by a statement that they have not been and will not be published in whole or in part in any other Journal.

3. **Summary** (2 copies) This should be a short summary of results and conclusions arranged in numbered paragraphs.

4. **Abstract** (2 copies) An abstract of about 100 words suitable for inclusion in an abstracting journal should be typed on a separate sheet, giving the author's name and address and the title of the paper.

5. **Figures** These should not be larger than A4 (206 x 294 mm) and may be in the form of original drawings, recorded tracings or high quality photographic prints made from them. Negative prints of kymograph tracings (black on white) should be provided, and both ordinates and abscissae should be calibrated. Lettering on figures should be inserted in pencil. Symbols which are to appear in figures or legends should be chosen from the following:

○ ● □ ■ △ ▲ ▽ ▼

Photographs and photomicrographs should be printed on glossy paper and should be larger than the size required for reproduction.

6. **Tables** These should not have more than 85 characters to a line (counting spaces between columns as 4 characters) and certainly not more than 110 characters to a line, unless absolutely unavoidable. **Figure legends** and **tables** should be typed on separate sheets of paper.

7. **References** These should be collected in alphabetical order at the end of the paper. They should include the authors' names, year of publication (in brackets), title of article, title of publication (abbreviated in accordance with the fourth edition of the *World List of Scientific Periodicals*), volume number and first and last page numbers. References to books should in addition, include the names of editors, the edition number, where appropriate, and the town of origin and name of publisher.

Two page proofs will be supplied, one of which may be retained by the authors The other should be corrected immediately and returned to the Press Editor. Corrections should be kept to a minimum.

Twenty-five reprints will be supplied to the authors free of charge. Additional reprints can be purchased. A reprint order form, which should be returned promptly, will be sent out with the proofs. No reprints of abstracts are supplied.

Short Communications These may be accepted if they merit priority publication and do not require revision. They may contain up to 1,200 words of text and only one figure or table. The summary should be a single paragraph. An abstract and statement should be included.

British Journal of Pharmacology

VOLUME 56 NUMBER 3 MARCH 1976

245 KOSS, M.C. & NAKANO, J. Reflex bradycardia and hypotension produced by prostaglandin $F_{2\alpha}$ in the cat

255 ASHFORD, J. & JONES, B.J. The effects of intra-amygdaloid injections of 6-hydroxydopamine on avoidance responding in rats

263 LEES, P. & SERRANO, L. Effects of azaperone on cardiovascular and respiratory functions in the horse

271 CLARKE, D.E. ROMANYSHYN, W.M. Blood pressure responses to adrenal field stimulation as a measure of adrenal catecholamine release

279 DORRIS, R.L. & SHORE, P.A. On the mechanism of action of clozapine on the adrenergic neurone

285 GUTMAN, Y. & MAZUR-RUDER, M. Rat uterus renin-like activity: effect of stimuli and hormones

293 HIMORI, N. & TAIRA, N. A method for recording smooth muscle and vascular responses of the blood-perfused dog trachea *in situ*

301 RIVERA-CALIMLIM LEONOR. Impaired absorption of chlorpromazine in rats given trihexyphenidyl

307 CURTIS, D.R., GAME, C.J.A. & LODGE, D. Benzodiazepines and central glycine receptors

313 DICK, W.C., GRENNAN, D.M. & ZEITLIN, I.J. Studies on the relative effects of prostaglandins, bradykinin, 5-hydroxytryptamine and histamine on the synovial microcirculation in dogs

317 CHAHL, LORIS A. & LADD, R.J. The effects of prostaglandins E_1, E_2 and $F_{2\alpha}$ on the cutaneous vasculature of the rat

323 BOWERY, N.G. & JONES, G.P. A comparison of γ-aminobutyric acid and the semi-rigid analogues 4-aminotetrolic acid, 4-aminocrotonic acid and imidazole-4-acetic acid on the isolated superior cervical ganglion of the rat

331 ANDERSON, A.A. & LEES, G.M. Investigation of occurrence of tolerance to bronchodilator drugs in chronically pretreated guinea-pigs

339P PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY, London, 17–19 December, 1975

Printed in Great Britain by The Whitefriars Press Ltd, London and Tonbridge

British Journal of Pharmacology

NOT TO BE LENT OUT

April 1976

Dup.
7.7.76

BRITISH COUNCIL

Edited for the British Pharmacological Society by

A.W. Cuthbert *(Chairman)* H.M. Adam *(Secretary)*
Margaret Day *(Press Editor)*
J.D. Stephenson *(Assistant Press Editor)*

A.M. Barrett
W.G. Bisset
T.B. Bolton
D.A. Brown
B.A. Callingham
T.J. Crow
F. de Matteis
C.T. Dollery
V. Eisen
E.W. Gill
A.F. Green
D.F. Hawkins
Margarethe Holzbauer
D.B. Hope
J.S. Kelly
G.M. Lees
G.P. Lewis
I.H.M. Main
E.K. Matthews
J.F. Mitchell
P.A. Nasmyth
M.W. Parkes
M. Sandler
A.P. Silverman
M.A. Simmonds
F.M. Sullivan
A. Ungar
R.A. Webster

Papers will be considered for publication on all aspects of drug action, including chemotherapy.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh EH8 9JZ. Authors should consult the instructions inside the back cover.

The British Journal of Pharmacology is published monthly by Macmillan Journals Ltd, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633. ISSN 0007-1188.

Annual subscription £36.00 ($90 USA and Canada). (Orders must be accompanied by remittance. Prices applicable only to orders received before 31st December 1976.)

Cheques should be made payable to Macmillan Journals Limited: Subscription Department, Brunel Road, Basingstoke, Hampshire RG21 2XS, England.

Enquiries concerning advertising space and rates should be addressed to: Advertisement Department, Macmillan Journals Limited, 4 Little Essex Street, London WC2R 3LF. Telephone: 01-836 6633.

Copyright © 1976 by the *British Journal of Pharmacology.* All rights of reproduction are reserved in respect of all papers, articles, illustrations, etc., published in this journal in all countries of the world.

Second class postage paid at New York, N.Y.
US Mailing Agent, Expeditors of the Printed Word, Ltd,
527 Madison Avenue, New York, NY 10022.

CHARLES C THOMAS • PUBLISHER

CLINICAL USE OF DRUGS IN RENAL FAILURE *by* **Robert J. Anderson**, *Univ. of Colorado, Denver;* **John G. Gambertoglio**, *Univ. of California, San Francisco; and* **Robert W. Schrier**, *Univ. of Colorado, Denver.* Patients with kidney disease generally receive more pharmacological agents than do individuals with normal kidney function. In addition to receiving more drugs, the patient with advanced renal failure may respond to a given dose of a pharmacological agent with respect to efficacy and adverse effects in a substantially different manner than the subject with healthy kidneys. This book examines these and other important considerations in the management of renal failure patients. Included in this book are antibacterial drugs, drugs used in the treatment of tuberculosis and fungal infections, drugs used in the treatment of gout, arthritis and inflammatory disorders, anticoagulants, antihypertensive agents, diuretics, antiarrhythmic drugs, immunosuppressive and antineoplastic agents, hypoglycemic drugs, analgesics and soporifics, and drugs used in neurology and psychiatry. A comprehensive drug reference, this book discusses the pharmacology, adverse effects, dialysis properties and appropriate dosage modifications required for renal failure patients. *'76, 246 pp., 1 il., 18 tables, $19.50*

CELLULAR PHARMACOLOGY OF EXCITABLE TISSUES *edited by* **Toshio Narahashi**, *Duke Univ. Medical Center, Durham, North Carolina. (12 Contributors)* Recent accomplishments in the field of cellular pharmacology are summarized in this multi-authored volume. In-depth discussion is given on the mechanisms of action of various drugs and chemicals on each excitable tissue including the central nervous system, ganglia, nerve fibers, skeletal, smooth and cardiac muscles. References are quoted in each chapter of this text. *'75, 552 pp., 120 il., 15 tables, $35.50*

ROLE OF CATECHOLAMINES IN CARDIOVASCULAR DISEASES: I Hypertension *by* **Budh Dev Bhagat**, *St. Louis, Missouri.* Basic physiology and pharmacology have been blended in this book in order to present an orderly approach to the practical management of patients with hypertensive disease. Schematic representation is used to explain the complex interaction of antihypertensive drugs with the mechanism for synthesis, storage, release and actions of norepinephrine. Extensive coverage is given to the most recent advances concerning the mechanisms of renin release, the metabolism of renin-angiotension and the relationship of the adrenergic nervous system and renin-angiotension. *'74, 176 pp. (7 x 10), 101 il., (10 in color), 18 tables, $17.50*

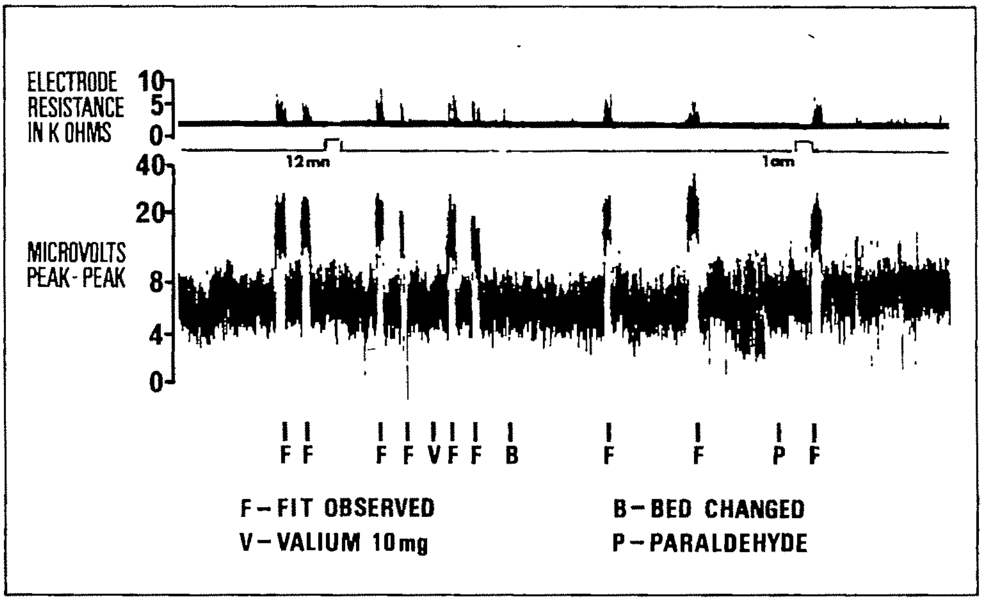
DRUG RESISTANCE IN ANTIMICROBIAL THERAPY *by* **E. J. L. Lowbury** *and* **G. A. J. Ayliffe**, *both of Medical Research Council, Birmingham, England.* In this book, two bacteriologists consider the biological mechanisms of resistance—mutation, transfer, selection—along with some of the associated biochemical changes. Biological mechanisms of resistance and associated biochemical changes are first considered. Chapters are devoted to the clinical interpretation of resistance, and to the organisms in which resistance has presented special problems. A section is included on resistant malaria. The concluding chapter presents general recommendations on antibiotic policies. *'74, 168 pp., 4 il., 4 tables, $16.75*

WHITE CELLS IN INFLAMMATION *edited by* **C. Gordon Van Arman**, *Merck Institute for Therapeutic Research, West Point, Pennsylvania. (11 Contributors)* Written by expert specialists, this text deals with the entrance of white cells into inflammation, platelets and permeability of blood vessels, cell migration, acute inflammation and white cells, eosinophils, white cells and gouty arthritis and other topics. This book was prepared so that all those working in inflammation may compare their concepts of etiology and treatment to those of the authors. *'74, 160 pp., 46 il., 12 tables, $14.50*

Orders with remittance sent by air, postpaid

301-327 East Lawrence Avenue • Springfield • Illinois • 62717 • USA

Epilepsy. Devices' CFM Helps Complete the Picture.



The Cerebral Function Monitor from Devices is making a major contribution to the understanding and treatment of epilepsy – helping to put together the complete picture.

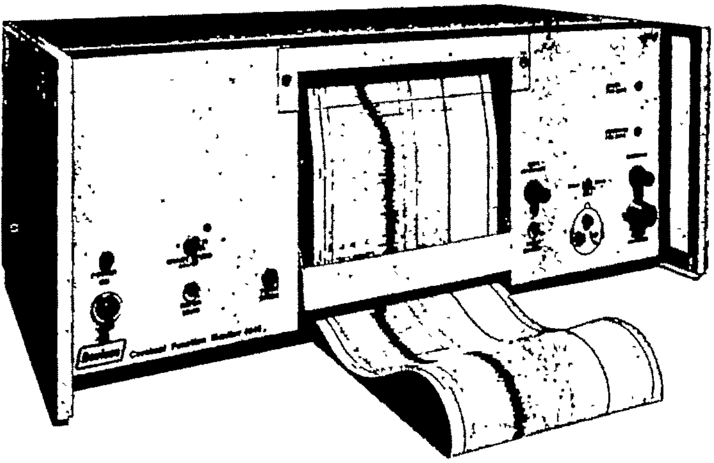
Accurate and reliable records of the frequency, intensity and duration of epileptic seizures can be obtained over long periods of time without the need for constant attention from skilled personnel. The CFM is easy to operate, simple to interpret and runs economically on only 6 cm/hr of chart paper.

Using a compressed and filtered signal from a pair of parietal electrodes, the CFM produces a thick band trace of cerebral activity. A second trace confirms the reliability of the cerebral recording by indicating the presence of any artefact such as mains interference or movement of the patient or electrodes. Scalp muscle activity is also picked up, which helps the clinician to determine whether a seizure is genuine or hysterical.

When curariform drugs are administered during treatment of status epilepticus, the CFM continues to record cerebral discharges even though clinical expression may not be apparent.

For full specification details and further examples of CFM wave-forms during epileptic seizure, please write or telephone:

Experience that shows.



Devices

Devices Limited, Instrument Division,
Hyde Way, Welwyn Garden City,
Hertfordshire AL7 3AP England.
Telephone: Welwyn Garden 28511

VOLUME 56(4) APRIL 1976

British Journal of Pharmacology

399 SMITH, D.F. Lithium orotate, carbonate and chloride: pharmacokinetics, polydipsia and polyuria in rats.

403 KAROUM, F. MAJCHROWICZ, E. & WYATT, R.J. Brain concentrations of biogenic amine metabolites in acutely treated and ethanol-dependent rats.

413 EGAN, SANDRA M., GRAHAM, J.D.P. & LEWIS, M.J. The uptake of tritiated Δ^1-tetrahydrocannabinol by the isolated vas deferens of the rat.

417 DRUMMOND, A.H. & GORDON, J.L. Uptake of 5-hydroxytryptamine by rat blood platelets and its inhibition by adenosine 5′-diphosphate.

423 MARCHAND, C. & NADEAU, D. The distribution and kinetics of sulphacetamide in leukaemic mice.

431 CARRUBA, M.O., GROPPETTI, A., MANTEGAZZA, P., VICENTINI, L. & ZAMBOTTI, F. Effects of mazindol, a non-phenylethylamine anorexigenic agent, on biogenic amine levels and turnover rate.

437 DUTTA, S., MARKS, B.H. & STEPHEN, P.M. The uptake and subcellular distribution of radiolabelled metabolites of digitoxin in the guinea-pig isolated perfused heart.

443 DOW, R.C. & LASZLO, I. Uptake of 5-hydroxytryptamine in different parts of the brain of the rabbit after intraventricular injection.

449 CARRIER, O., JR., & HESTER, R.K. The relationship between calcium and increased sensitivity of rabbit aortae four hours after reserpine.

457 MICHELSON, M.J. & SHELKOVNIKOV, S.A. Isotonic and isometric responses of different tonic muscles to agonists and antagonists.

469 FERREIRA, S.H., HERMAN, A.G. & VANE, J.R. Prostaglandin production by rabbit isolated jejunum and its relationship to the inherent tone of the preparation.

479 MAGOS, L. The effects of dimercaptosuccinic acid on the excretion and distribution of mercury in rats and mice treated with mercuric chloride and methylmercury chloride.

485 GRAY, J.A., LÜLLMANN, H., MITCHELSON, F. & REIL, G.-H. Stereoselective binding in cardiac tissue of the enantiomers of benzetimide, an antimuscarinic drug.

SHORT COMMUNICATIONS

491 KUMAR, A., SHARMA, H.L. & SHARMA, V.N. Effect of SAS (A new 10,*N*-acylaminophenothiazine) on gastric secretion and ulceration in rats.

494 CHIPMAN, P. & GLOVER, W.E. Histamine H_2-receptors in the human peripheral circulation.

Br. J. Pharmac. (1976), **56**, 399–402

LITHIUM OROTATE, CARBONATE AND CHLORIDE: PHARMACOKINETICS, POLYDIPSIA AND POLYURIA IN RATS

D.F. SMITH
Psychopharmacology Research Unit, Psychiatric Hospital, Risskov, Denmark

1 The pharmacokinetics of the lithium ion administered as lithium orotate were studied in rats. Parallel studies were carried out with lithium carbonate and lithium chloride.

2 No differences in the uptake, distribution and excretion of the lithium ion were observed between lithium orotate, lithium carbonate and lithium chloride after single intraperitoneal, subcutaneous or intragastric injections (0.5–1.0 mEq lithium/kg) or after administration of the lithium salts for 20 days in the food.

3 The findings oppose the notion that the pharmacokinetics of the lithium ion given as lithium orotate differ from lithium chloride or lithium carbonate.

4 Polyuria and polydipsia developed more slowly in rats given lithium orotate than in those given lithium carbonate or lithium chloride, perhaps due to an effect of the orotate anion.

Introduction

Lithium orotate was recently introduced as a drug by Nieper (1973a) who used it in clinical trials in the hope of applying the principle of directed electrolyte transport in lithium therapy. His studies on calcium orotate and magnesium orotate indicated that these salts pass through the cell membrane in undissociated form and release the respective ions only at the site of membranes of cytoplasmic structures (Nieper, 1969; 1970; 1973b). Nieper assumed that lithium orotate also would be taken up in the undissociated form specifically into the tissues of the central nervous system whereupon the lithium ion would be liberated within the cells (Nieper, 1973a).

To date, no detailed information is available concerning the uptake, distribution and excretion of lithium orotate. The present study was carried out primarily to investigate the pharmacokinetics of the lithium ion when administered as lithium orotate. In addition, the effect of long-term administration of lithium orotate on water intake and urine output in rats was investigated. Parallel investigations were carried out with lithium carbonate and lithium chloride.

Methods

Male albino Wistar rats weighing 250–300 g were housed in a thermostatically controlled room (23°C) on a 12 h light-dark cycle (lights on 8 h 00 min to 20 h 00 min) with rat chow pellets and tap water freely available for at least 3 weeks before the experiments.

Short-term experiments

Serum lithium concentrations and urinary lithium excretion were studied in 9 rats given an intraperitoneal, subcutaneous or intragastric injection of 0.5 mEq lithium/kg body weight as 0.05 M lithium orotate (LiOr), 0.05 M lithium chloride (LiCl) or 0.025M lithium carbonate (Li_2CO_3) at 10 h 00 min. Blood samples were taken at 10 h 40 min and 14 h 30 min under ether anaesthesia from the rat tails. Urine was collected between 11 h 00 min and 14 h 00 min as described in detail previously (Smith, 1974). Each rat was tested 5 times at 3–4 day intervals and received a different treatment prior to each test. The lithium concentration in the serum and urine samples was determined by flame photometry (Amdisen, 1967).

The distribution of lithium was examined in 8 rats killed at 15 h 15 min, 7 h after an intragastric injection (1 mEq lithium/kg) of either 0.05 M LiOr or 0.025 M Li_2CO_3. The lithium concentration in tissues, red blood cells and plasma was determined by flame photometry (Schou, 1958; Amdisen, 1967).

Long-term experiments

Water intake, urine output and the distribution of lithium were studied in 16 rats randomly divided into 4 equal groups and given free access to wet mash diet (Thomsen, 1970) containing either no lithium or LiOr, Li_2CO_3 or LiCl for 20 days. The lithium concentration in the food was increased by 15 mEq/kg dry wt at 4 day intervals until the concentration of lithium was 60 mEq/kg dry wt; it was kept at this level thereafter. Tap water intake was measured daily. Blood samples were taken into heparinized tubes periodically under ether anaesthesia from the rat tails. On the 20th day, the volume of urine excreted by the rats was measured in individual metabolism cages without food or water present from 10 h 00 min to 14 h 00 min. The rats were killed thereafter and the lithium concentration in the tissues and blood was determined by flame photometry (Amdisen, 1967; Schou, 1958).

Results

Short-term experiments

The results presented in Table 1 show that the serum lithium levels and the amounts of lithium excreted in the urine obtained after intraperitoneal, subcutaneous or intragastric administration of LiOr did not differ significantly in any respect from the results obtained with Li_2CO_3 or LiCl. The serum lithium level 40 min after intragastric injection of the lithium salts was significantly less than after intraperitoneal or subcutaneous injections ($P < 0.05$). A significant decline occurred in the serum lithium level during the test after intraperitoneal or subcutaneous injections

Table 1 Serum lithium concentration at 40 min and 4.5 h postinjection and urinary lithium excretion from 1 to 4 h postinjection in rats given an intraperitoneal (i.p.), subcutaneous (s.c.) or intragastric (i.g.) injection (0.5 mEq lithium/kg body wt.) of lithium orotate, lithium carbonate or lithium chloride

		Serum lithium concentration (mEq/l)		*Renal lithium excretion (µEq kg^{-1} h^{-1})*
		40 min	*4.5 h*	
Lithium orotate	i.p.	0.40 ± 0.02	0.20 ± 0.03	30.0 ± 2.4
	s.c.	0.45 ± 0.02	0.23 ± 0.10	30.7 ± 5.1
	i.g.	0.28 ± 0.02	0.22 ± 0.02	31.4 ± 3.2
Lithium carbonate	i.p.	0.45 ± 0.03	0.21 ± 0.01	31.8 ± 5.7
	s.c.	0.52 ± 0.06	0.23 ± 0.03	31.4 ± 5.1
	i.g.	0.28 ± 0.01	0.22 ± 0.02	32.8 ± 4.6
Lithium chloride	i.p.	0.39 ± 0.04	0.18 ± 0.03	29.7 ± 5.2
	s.c.	0.44 ± 0.04	0.17 ± 0.02	30.2 ± 2.7
	i.g.	0.28 ± 0.05	0.20 ± 0.04	29.6 ± 2.3

Values are means ± s.d. for 5 rats

Table 2 Lithium concentration in blood and tissues 7 h after a stomach load of lithium orotate or lithium carbonate (1 mEq/kg body wt.)

	Lithium concentration (mEq/kg wet wt; mEq/l)	
Tissue	*Lithium orotate*	*Lithium carbonate*
Brain (whole)	0.11 ± 0.03	0.10 ± 0.01
Liver (middle lobe)	0.12 ± 0.01	0.11 ± 0.01
Muscle (gastrocnemius)	0.20 ± 0.01	0.19 ± 0.01
Lung	0.22 ± 0.02	0.22 ± 0.02
Heart (whole)	0.27 ± 0.03	0.29 ± 0.01
Red blood cells	0.36 ± 0.05	0.32 ± 0.08
Kidney	0.36 ± 0.05	0.37 ± 0.06
Plasma	0.44 ± 0.04	0.44 ± 0.03

Values are means ± s.d. for 4 rats.

($P < 0.05$). The route of administration of LiOr, Li_2CO_3 and LiCl had no significant effect on the amount of lithium excreted in the urine.

The results presented in Table 2 show that the concentration of lithium in the blood and tissues in rats given LiOr did not differ significantly in any respect from the levels obtained in animals given Li_2CO_3. Lithium was not uniformly distributed throughout all tissues after short-term administration; the lowest concentrations were obtained in the brain and liver and the highest levels were in the plasma and kidney.

Long-term experiments

The data in Table 3 show that the plasma lithium concentration in rats given LiOr in their food did not differ significantly in any respect from the groups given Li_2CO_3 or LiCl in their food. As the concentration of lithium in the food was increased, the plasma lithium concentration rose similarly in all the groups.

The data in Table 4 show that the concentration of lithium in the blood and tissues in rats given LiOr in their food did not differ significantly in any respect from the levels obtained in animals given Li_2CO_3 or LiCl in their food. Lithium was not uniformly distributed throughout all tissues after long-term administration; the lowest concentrations were obtained in the liver and the highest levels were in the kidney and muscle.

Figure 1 shows that the water intake increased significantly above control values in all groups given lithium; it was significantly higher than control levels after 9 days of treatment in rats given Li_2CO_3 or LiCl ($P < 0.05$), while 12 days of treatment with LiOr were required to produce a statistically significant ($P < 0.05$) increase in water intake. Within the experimental period, the water intake in rats given LiOr did not reach the level obtained in animals given Li_2CO_3 or LiCl.

Table 3 Plasma lithium concentration during long-term administration of increasing concentrations of lithium orotate, lithium carbonate or lithium chloride in the food

		Plasma lithium concentration (mEq/l)		
Lithium concentration in food (mEq/kg dry wt)	*Days*	*Lithium orotate*	*Lithium carbonate*	*Lithium chloride*
15	1–4	0.23±0.03	0.21±0.01	0.22±0.02
30	5–8	0.38±0.03	0.34±0.03	0.38±0.02
45	9–12	0.47±0.04	0.52±0.04	0.48±0.07
60	13–15	0.54±0.06	0.58±0.06	0.51±0.03

Values are means ± s.d. for 4 rats.

Table 4 Lithium concentration in blood and tissues after administration of lithium orotate, lithium carbonate or lithium chloride in the food for 20 days

	Lithium concentration (mEq/kg wet wt; mEq/l)		
Tissue	*Lithium orotate*	*Lithium carbonate*	*Lithium chloride*
Liver (middle lobe)	0.34±0.07	0.30±0.01	0.31±0.08
Plasma	0.63±0.11	0.65±0.08	0.64±0.07
Red blood cells	0.66±0.06	0.68±0.10	0.59±0.09
Lung	0.67±0.06	0.67±0.11	0.58±0.06
Brain (whole)	0.68±0.05	0.67±0.03	0.67±0.[illegible]8
Heart (whole)	0.74±0.10	0.72±0.04	0.70±0.08
Muscle (gastrocnemius)	0.76±0.12	0.78±0.10	0.83±0.13
Kidney	1.01±0.21	0.92±0.06	0.95±0.04

The lithium concentration in the food was 60 mEq/kg dry wt. for the last 8 days of treatment. Values are means ± s.d. for 4 rats.

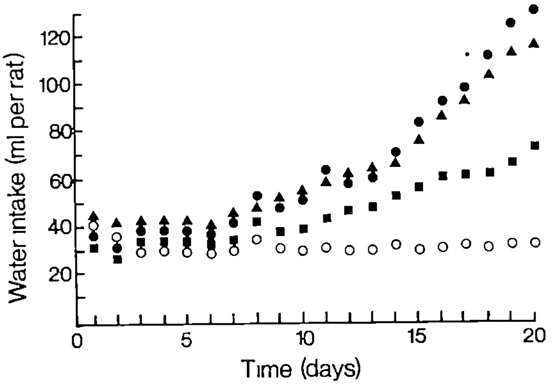


Figure 1 Mean daily water intake in rats given either no lithium (○) or increasing concentrations of lithium orotate (■), lithium carbonate (▲) or lithium chloride (●) in the food. The concentration of lithium in the food was 15 mEq/kg dry wt from day 1 to 4, 30 mEq/kg dry wt. from day 5 to 8, 45 mEq/kg dry wt from day 9 to 12, and 60 mEq/kg dry wt. from day 13 to 20.

On the 20th day, the control group excreted 3.5 ± 0.7 ml of urine during the test. The urine volume in the group given LiCl (11.1 ± 2.9 ml) was significantly greater ($P<0.05$) than in the control group as well as in the group given LiOr (6.9 ± 3.0 ml). The urine output of rats given Li_2CO_3 (9.9 ± 4.1 ml) was significantly greater than the control level ($P<0.05$) but did not differ significantly from the groups given LiCl or LiOr. The urine output in the control group and the group given LiOr did not differ significantly.

Discussion

No differences were observed between LiOr, Li_2CO_3 and LiCl in lithium absorption, distribution and urinary excretion after short-term or long-term administration. The features of lithium pharmacokinetics previously established using LiCl and Li_2CO_3, such as more rapid uptake of lithium after intraperitoneal injection than after intragastric administration (Morrison, Pritchard, Braude & D'Aguanno, 1971), higher lithium concentrations in serum than in brain soon after short-term lithium administration (Schou, 1958; Ebadi, Simmons, Hendrickson & Lacy, 1974), higher lithium concentrations in kidney and brain than in liver after long-term lithium administration (Birch & Hullin, 1972), and higher lithium concentrations in red blood cells than in plasma during prolonged administration of lithium (Smith, 1975) also were observed in the present study of LiOr. Thus, the findings offer no support whatsoever for the assumption that the pharmacokinetics of lithium ions given as LiOr differ from LiCl or Li_2CO_3 (Nieper, 1973a).

Polydipsia and polyuria occurred during long-term administration of LiOr, LiCl and Li_2CO_3. There was a tendency, however, for the onset of polydipsia and polyuria to be delayed during LiOr treatment compared to LiCl and Li_2CO_3; an unexpected finding since the pharmacokinetic studies showed no differences between the groups given LiOr, LiCl or Li_2CO_3 in the concentrations of lithium in blood and tissues. Although the mechanism responsible for the difference is not known, it might be due to an effect of the orotate anion.

The author thanks Nadrol-Chemie-Pharma, Osnabrück, Germany for the generous supply of lithium orotate; Mrs Marianne Jensen for technical assistance; Aarhus University and Carl P. Petersens Fund for financial support.

References

AMDISEN, A. (1967). Serum lithium determinations for clinical use. *Scand. J. clin. Lab. Invest.*, **20**, 104–108.

BIRCH, N J & HULLIN, R.P. (1972). The distribution and binding of lithium following its long-term administration. *Life Sci.*, **11**, 1095–1099.

EBADI, M.S, SIMMONS, V.J, HENDRICKSON, M.J & LACY, P.S. (1974). Pharmacokinetics of lithium and its regional distribution in rat brain. *Eur. J. Pharmac.*, **27**, 324–329.

MORRISON, J M Jr., PRITCHARD, H.D., BRAUDE, M.C. & D'AGUANNO, W. (1971). Plasma and brain lithium levels after lithium carbonate and lithium chloride administration by different routes in rats. *Proc. Soc. exp. Biol.*, **137**, 889–892.

NIEPER, H.-A. (1969). The anti-inflammatory and immune inhibiting effect of calcium orotate on bradytrophic tissue. *Agressologie*, **10**, 349–354.

NIEPER, H -A. (1970). Recalcification of bone metastases by calcium diorotate. *Agressologie*, **11**, 495–502.

NIEPER, H.-A. (1973a). The clinical applications of lithium orotate. A two years study. *Agressologie*, **14**, 407–411.

NIEPER, H.-A. (1973b). Klinische Wirkung von Calcium-Diorotat auf Knorpelgewebe. Spezifische Funktion in Abhangigkeit vom Pentosen-Stoffwechsel bradytropher Gewebe. *Z. Geriat.*, **3**, 73–82.

SCHOU, M (1958). Lithium studies. 3. Distribution between serum and tissues. *Acta pharmac. (Kbh.)*, **15**, 115–124.

SMITH, D F. (1974). Renal lithium clearance during dehydration and rehydration with water or 0.9% NaCl in the rat. *Acta physiol scand.*, **90**, 427–430.

SMITH, D.F. (1975). Biogenic amines and the effect of short-term lithium administration on open field activity in rats. *Psychopharmacologia (Berl.)*, **41**, 295–300.

THOMSEN, K. (1970). Lithium-induced polyuria in rats. *Int. Pharmacopsychiat.*, **5**, 233–241.

(Received June 2, 1975.
Revised October 13, 1975.)

Br. J. Pharmac. (1976), **56**, 403–411

BRAIN CONCENTRATIONS OF BIOGENIC AMINE METABOLITES IN ACUTELY TREATED AND ETHANOL-DEPENDENT RATS

F. KAROUM & R.J. WYATT
Laboratory of Clinical Psychopharmacology, Saint Elizabeths Hospital
SMR, IRP, NIMH Washington, D C. 20032, U.S A.

E. MAJCHROWICZ
Laboratory of Alcohol Research, NIAAA, Saint Elizabeths Hospital,
Washington, D.C. 20032, U.S A.

1 Mass fragmentography was used to measure whole brain concentrations of some of the major metabolites of tyramine, octopamine, dopamine and noradrenaline in acutely treated and in ethanol-dependent rats.

2 Treatments with ethanol, either acutely or chronically, failed to alter significantly brain concentration of *p*-hydroxyphenylacetic and *p*-hydroxymandelic acid (metabolites derived from tyramine and octopamine respectively). The effect on catecholamine metabolites was marked and therefore suggests that ethanol is selective in its effect on central metabolism of biogenic amines.

3 Acute ethanol treatment significantly increased brain concentration of homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxy-4-hydroxyphenylglycol (MHPG). Vanilmandelic acid (VMA) was not affected. All four metabolites (HVA, DOPAC, MHPG and VMA) were increased in the brains of rats rendered dependent on ethanol while still intoxicated (blood ethanol levels above 200 mg/dl). In ethanol-dependent rats undergoing ethanol withdrawal syndrome (no ethanol present in blood), the brain concentrations of HVA and DOPAC were normal while those of MHPG and VMA continued to be elevated.

4 From the decline in the concentrations of HVA and DOPAC after 50 mg pargyline/kg in control rats and rats acutely treated with ethanol, it was concluded that ethanol has no effect on the transport of phenolic acids across the blood brain barrier.

5 No reversal in the metabolism of catecholamines from an oxidative to a reductive pathway, analogous to that produced by ethanol in the periphery, could be established in the brain.

6 The increase in catecholamine metabolite concentrations after ethanol treatment, either acute or chronic, were interpreted as manifestations of increases catecholamine turnover.

Introduction

The involvement of biogenic amines in the mediation of central and peripheral effects of ethanol has been postulated by several authors (for a review see Feldstein, 1971; Truitt & Walsh, 1971; Lahti, 1975). Most studies on the effect of ethanol on the metabolism of biogenic amines have been confined to the peripheral sympathetic nervous system where a reversal from an oxidative to a reductive pathway of several amines is well documented (Smith & Gitlow, 1967; Davis, Brown, Huff & Cashaw, 1967a,b; Tacker, Creaven & McIssac, 1970; Bonham Carter, Karoum, Sandler & Youdim, 1970; Ogata, Mendelson, Mello & Majchrowicz, 1971). Attempts to demonstrate changes in biogenic amine metabolites in the central nervous system following the administration of ethanol have been limited to 5-hydroxytryptamine (von Wartburg, 1962; Lahti & Majchrowicz, 1967, 1969; Feldstein & Sidel, 1969; Tyce, Flock, Taylor & Owen, 1970; Tabakoff, Bulat & Anderson, 1975; Tabakoff, Ritzman & Boggan, 1975). In this paper we describe a study on the brain levels of metabolites derived from tyramine, octopamine, dopamine and noradrenaline in rats either treated acutely with ethanol or rendered physically dependent upon ethanol.

Methods

Animals

Male Sprague-Dawley rats weighing between 200 and 300 g were used. After delivery, the animals were allowed to acclimatize to animal house conditions for two to four days. The animals had unlimited access to food and water throughout the entire experimental period.

Acute experiments

In two sets of experiments, ethanol was administered intragastrically to 12 rats in a dose of 5 g/kg as a 20% (w/v) aqueous solution. Control animals were given 5 ml 0.9% w/v NaCl solution (saline). Three hours later the rats were decapitated and their brains removed and frozen on dry ice. In one set of experiments, the brains were used for the assay of the acidic metabolites (*p*-hydroxyphenylacetic (PHPA), *p*-hydroxymandelic (PHMA), dihydroxyphenylacetic (DOPAC), homovanillic (HVA) and vanilmandelic (VMA) acids) and the other for the assay of 3-methoxy-4-hydroxyphenylglycol (MHPG).

In another set of experiments designed to study the effect of ethanol on the transport mechanism of HVA and DOPAC across the blood brain barrier, 25 rats were given ethanol as described above. One hour later, pargyline (50 mg/kg), a monoamine oxidase (MAO) inhibitor, was given intraperitoneally. Groups of five rats were decapitated immediately and after intervals of 30, 60, 120 and 180 minutes. Control rats were given saline and treated with pargyline exactly as described for the ethanol-treated rats.

Ethanol-dependent rats

Physical dependence upon ethanol was induced in 20 rats by intragastric administration of a 20% (w/v) ethanol solution at 9–15 g/kg daily in three to five fractional doses over four days. Ten to 15 h after the last dose of ethanol, the rats were separated into two groups depending upon the development of the withdrawal syndrome. The rats in the first group showed signs of varying severity during the prodromal detoxication phase of withdrawal. These rats had blood ethanol levels higher than 200 mg/dl. The second group of rats showed overt signs and responses of an ethanol withdrawal syndrome (Majchrowicz, 1973; 1975). During this phase, little or no ethanol was found in the blood. The ethanol withdrawal phase was characterized by the onset of the withdrawal signs and responses reminiscent of the tremulous, spastic and convulsive stages of ethanol-dependence observed in man (Victor & Adams, 1953; Mendelson & LaDou, 1964). In two sets of experiments five rats from each of the above two groups of ethanol-dependent as well as control rats were decapitated and their brains frozen on dry ice. One set of animals was used for the assay of the acidic metabolites and the other for the assay of MHPG.

Equipment

Combined gas chromatography-mass spectrometry was carried out on a Finnigan Model 3000D

Table 1 Atomic mass units of fragments employed for mass fragmentography of biogenic amine metabolites

Metabolite	*Derivative*	*m/e used for mass fragmentography (origin of fragment)*	*Relative retention time**
p-Hydroxyphenylacetic acid (PHPA)	Methyl ester/pentafluoropropionyl	253 (M^+–$COOCH_3$)/312 (M^+)	1.0
p-Hydroxymandelic acid (PHMA)	Methyl ester/pentafluoropropionyl	387 (M^+–$COOCH_3$–CO)/415 (M^+–$COOCH_3$)	1.0
Homovanillic acid (HVA)	Methyl ester/pentafluoropropionyl	283 (M^+–$COOCH_3$)/342 (M^+)	1.78
Dihydroxyphenylacetic acid (DOPAC)	Methyl ester/pentafluoropropionyl	387 (M^+–$COOCH_3$–CO)/415 (M^+–$COOCH_3$)	1.38
Vanilmandelic acid (VMA)	Methyl ester/pentafluoropropionyl	445 (M^+–$COOCH_3$)/417 (M^+–CH_3OH)	1 50
3-Methoxy-4-hydroxyphenylglycol (MHPG)	Pentafluoropropionyl	*311 (M^+–$OCOHC_2F_5$–COC_2F_5)/458 (M^+–$OCOHC_2F_5$)†	1 22

* Retention time relative to PHPA. † This ion is produced by the transfer of a β hydrogen onto the keto oxygen of the acyl group through a two-step hydrogen rearrangement reaction

Quadrupole gas chromatograph mass spectrometer employing an 8 ft, ⅛ inch i.d. 3% SE-54 steel column. The oven temperature was maintained at 200°C or 195°C depending on the type of analysis. The fragments selected for mass fragmentography are summarized in Table 1.

Reagents

Pentafluoropropionic anhydride and pentafluoropropionic imidazole were obtained from Pierce Chemical Company, Rockford, Illinois; SE-54 (GP91C silicon Gum rubber) was obtained from Analab, North Haven, Connecticut. 'Lipopure' methanol was purchased from Applied Science Laboratories, Inc., State College, Pennsylvania. Deuterated reagents used for the synthesis of deuterated compounds came from Merck, Sharpe & Dohme, Canada, Ltd., Montreal, Quebec. All other reagents and compounds used were of the highest purity available. Procedure for the synthesis of the different deuterated isomers are published elsewhere (Karoum, Gillin & Wyatt, 1975a, Karoum, Gillin, Wyatt & Coster, 1975b).

Derivatization

Acidic metabolites were first methylated with 100 μl 20% HCl in methanol (prepared by adding 1 ml acetylchloride dropwise to 4 ml 'Lipopure' methanol; acetylchloride quantitatively reacts with methanol to form HCl and methyl acetate.) After 5 min at room temperature, the mixture was dried under a gentle stream of N_2. The methyl ester was next acylated with 50 μl 10% pentafluoropropionyl imidazole in ethyl acetate by heating at 70°C for 10 minutes. One μl of the derivative was injected.

Three-methoxy-4-hydroxyphenylglycol was derivatized in a mixture of 100 μl ethyl acetate and 100 μl pentafluoropropionic anhydride as previously described (Karoum, LeFevre, Bigelow & Costa, 1973) and the pentafluoropropionyl derivative reconstituted in 50 μl ethyl acetate.

The use of pentafluoropropionyl imidazole in the preparation of the methyl ester/pentafluoropropionyl derivatives of the acidic metabolites consistently gave a higher yield of products than did the pentafluoropropionic anhydride. The opposite was true for MHPG.

Processing of brain tissue

The brains were weighed, individually homogenized in 10 ml 1 N HCl, centrifuged at 20,000 *g* for 15 min, and the clear supernatant used for the analysis of the acidic metabolites.

For the alcoholic metabolites, brains were homogenized individually in 6 ml 0.2 M $ZnSO_4$, to which 6 ml 0.2 M $Ba(OH)_2$ was added. The mixture was further homogenized for 1 min and centrifuged at 20,000 *g* for 15 minutes. The clear supernatants were used for analysis.

Assay of acidic metabolites: vanilmandelic acid, homovanillic acid, p*-hydroxyphenylacetic acid, dihydroxyphenylacetic acid and* p*-hydroxymandelic acid*

Into two 45 ml round-bottom glass centrifuge tubes, 2 ml of the clear supernatant obtained from the brain homogenate was introduced followed by the addition of 100 μl of a mixture of appropriate deuterated isomers (5–20 ng) (VMA-d_3, HVA-d_2, PHPA-d_4, DOPAC-d_5, PHMA-d_2). Internal standards (5–50 ng) of the non-deuterated metabolites were also added to one duplicate. The amount of internal standard used was about the same as that expected in the sample analyzed. The mixture in each tube was saturated with anhydrous Na_2SO_4 and then extracted twice with 10 ml ethyl acetate. Aliquots (8 and 10 ml) of the ethyl acetate extracts were respectively transferred, combined and evaporated under vacuum. The dried residue thus obtained was reconstituted in 0.3 ml ethyl acetate; 0.2 ml transferred into 1 ml microflex tubes (Kontes Glass Company, Evanston, Illinois); the 0.2 ml aliquot dried under N_2 and the metabolites in the dried residue converted to their methyl ester/pentafluoropropionyl derivatives. The deuterated isomers were used to correct for any variance that may have been introduced during the extraction and preparation of the non-deuterated metabolites as will be described later.

Assay of unconjugated 3-methoxy-4-hydroxyphenylglycol (MHPG)

Two ml of the clear supernatant obtained from the brain homogenate was mixed in duplicate with 1 ml 1M acetate buffer pH 6.2. To one duplicate, 10 ng of MHPG from a freshly prepared diluted solution was added followed by the addition of 10 ng of deuterated MHPG (MHPG-d_3) to both tubes. The duplicates were then saturated with anhydrous Na_2SO_4, extracted and processed as described for the acidic metabolites, except that the pentafluoropropionyl derivative was prepared.

Assay of total 3-methoxy-4-hydroxyphenylglycol (MHPG)

One ml of the clear supernatant from the brain homogenate was mixed in duplicate with 1 ml 1 M acetate buffer pH 6.2. To one duplicate, 100 μl of diluted MHPG-sulphate solution (corresponding to 20 ng MHPG) was added and the duplicates incubated for 20 h at 40°C with 0.2 ml of a high molecular fraction of glusulase (Karoum *et al.*, 1973). After hydrolysis, MHPG-d_3 (10 ng) was added to all tubes and the samples were processed exactly as described for the free metabolites. A sample blank containing 2 ml 1 M acetate buffer was included and used to correct for the presence of MHPG (usually about 2 ng/ml) in the glusulase.

Quantification procedure

As described earlier, each brain was analyzed in duplicate (samples A and B) by assaying equal volumes of the clear supernatant obtained from the brain homogenate. To these duplicates (A and B), equal amounts of a deuterated standard were added. An internal non-deuterated standard was also added to one duplicate (A). After processing the samples, the mass fragmentograms of the duplicate samples (A and B), the peak heights of the non-dueterated metabolite and of the corresponding deuterated standard were measured. Metabolite peak heights were expressed as a proportion of the peak height of the corresponding deuterated metabolite. Endogenous metabolite levels were calculated from the change in this ratio due to the addition of the non-deuterated standard to the duplicate (A).

Assay of blood ethanol

Ethanol levels were determined in mixed arterio-venous blood taken immediately after decapitation using an automated adaptation (Majchrowicz, 1971) of the gas chromatographic method of Roach & Creaven (1968).

Results

The concentrations of the various metabolites and blood ethanol levels in the acutely treated rats are shown in Table 2, and those for the ethanol-dependent rats in Table 3. With the exception of one rat that had a blood ethanol concentration of 78 mg/dl, there was no ethanol present in the blood of rats undergoing an ethanol withdrawal syndrome.

The concentrations of PHPA and PHMA, which are metabolites of tyramine (Ewins & Laidlaw, 1910; Schayer, 1953; Armstrong, Shaw & Wall, 1956; Spector, Melmon, Lovenberg & Sjoerdsma, 1963) and octopamine (Armstrong *et al.*, 1956; Pisano, Creveling & Udenfriend, 1960) respectively, were not significantly affected by ethanol in any of the treated rats. However, the concentrations of DOPAC and HVA were significantly increased both in the acutely treated and in the ethanol-dependent rats while still intoxicated. After the clearance of ethanol from the blood, both DOPAC and HVA reverted to normal concentrations in the ethanol-dependent rats. In contrast, MHPG was elevated in the acutely treated as well as in the ethanol-dependent rats irrespective of whether they were intoxicated or undergoing a

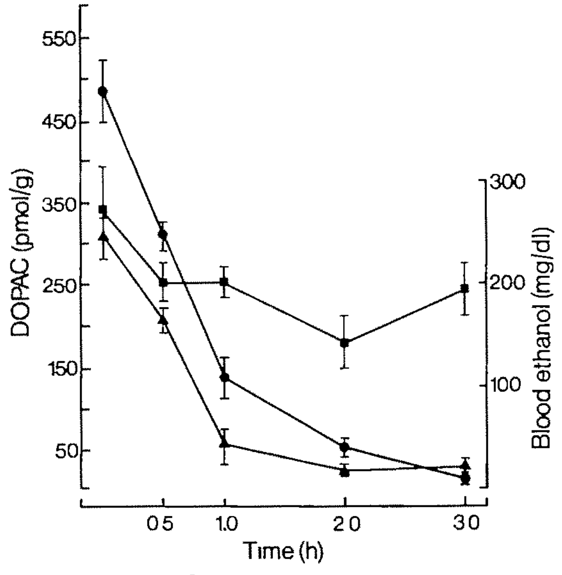


Figure 1 Brain concentrations (pmol/g) of dihydroxyphenylacetic acid (DOPAC) after pargyline. Ethanol (5 g/kg) was given intragastrically 1 h before pargyline (50 mg/kg). Control (▲); intoxicated rats (●), blood ethanol (■) All means for the ethanol-treated rats were significantly higher than those for the controls ($P < 0.05$)

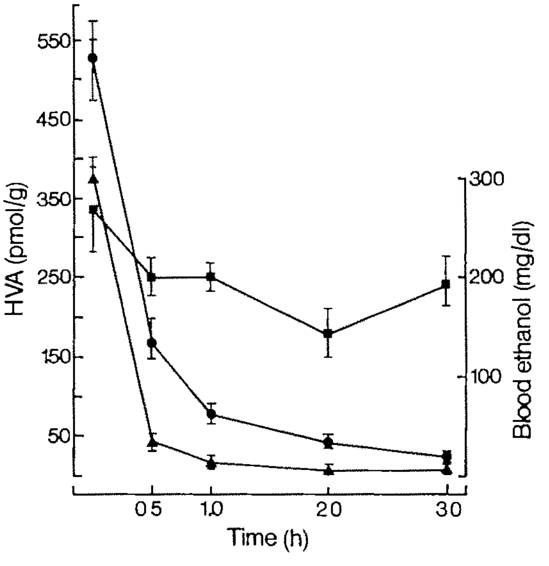


Figure 2 Brain concentrations (pmol/g) of homovanillic acid (HVA) after pargyline (50 mg/kg). Ethanol was given as described in Figure 1 Control (▲); intoxicated rats (●), blood ethanol (■). All means of the ethanol-treated rats were significantly higher than those of the control.

Table 2 Whole rat brain amine metabolites in acute ethanol-treated and control rats*

Description	*No. of rats*	*PHPA*	*PHMA*	*VMA*	*Free MHPG*	*Total MHPG*	*Total MHPG/VMA*	*HVA*	*DOPAC*	*Blood ethanol*
Controls	6	—	18.4±1.6	10.1±2.5	69.6±3.2	479±49	46.2±12	445±24	369±20	0
Treated†	12	—	19.0±2.1	6.5±1.0	99.9±12.5	679±32	103.7±49	857±33.5	556±20	193.4±24
P value	—	—	NS	NS		*P*<0.05	*P*<0.005	*P*<0.005	*P*<0.001	

PHPA=*p*-hydroxyphenylacetic acid; PHMA=*p*-hydroxymandelic acid; VMA=3-methoxy-4-hydroxyvanilmandelic acid, MHPG=3-methoxy-4-hydroxyphenylglycol; HVA=homovanillic acid; DOPAC=dihydroxyphenylacetic acid. * Results are expressed in pmol/g tissue as their mean±s.e. mean. † Rats were given intragastrically 5 g of ethanol in 20% (w.v.) solution per/kg of body weight and decapitated 3 h later.

Table 3 Whole rat brain amine metabolites in ethanol-dependent and control rats*

Description	*No. of rats*	*PHPA*	*PHMA*	*Total MHPG*	*Total MHPG/VMA*	*VMA*	*HVA*	*DOPAC*	*Blood ethanol*
A. Controls	5	164±26	15.2±1.6	414±49	20.8±2	18.6±2	510±39	407±32	—
B. Intoxicated†	5	230±26	22.8±3.8	597±61	13.4±3.9	52.5±11.1	659±42.8	630±67	230±21
C. Withdrawn‡	5	158±5.9	15.2±2.1	804±68	30±12	52.5±12	516±21	429±27	§
P value, A/B		NS	NS	*P*<0.05	NS	*P*<0.02	*P*<0.025	*P*<0.02	—
P value, A/C		NS	NS	*P*<0.001	NS	*P*<0.005	NS	NS	—
P value, B/C		NS	NS	*P*<0.05	NS	NS	*P*<0.02	*P*<0.025	—

Abbreviations as in Table 2. * Results are expressed in pmol/g tissue as their mean±s.e. mean. † Rats were given intragastrically 20% (w/v) ethanol at 9 to 15 g/kg in three to five fractional doses daily for four days and were decapitated during the prodromal detoxication phase of the withdrawal period while intoxicated. ‡ Rats were treated as described for the intoxicated group (†) but were decapitated while showing overt withdrawal signs (see text). § One rat among this group had a blood ethanol of 78 mg/dl.

withdrawal syndrome. The concentration of VMA was elevated only in the ethanol-dependent rats. It is worth mentioning that 3-methoxy-4-hydroxyphenylethanol (MHPE) (the alcoholic counterpart of HVA) was semiquantitatively evaluated in some of the acutely and chronically treated rats and compared with controls. It was found that brain concentration of this metabolite was not markedly affected by ethanol ingestion. Semiquantitative evaluation of MHPE was carried out because the gas chromatographic column employed here does not allow accurate measurement of this metabolite (Karoum, Gillin, Wyatt & Coster, 1975b).

When the concentrations of HVA plus DOPAC and MHPG plus VMA were compared in the control and acutely treated rats, the percentage increase was found to be 53.5 and 58.8, respectively. The percentage increases between the control and ethanol-dependent rats while still intoxicated were 40.8 and 50.4, respectively.

The changes in the concentration of brain HVA and DOPAC in the control and acutely treated rats following pargyline (50 mg/kg) are summarized in Figures 1 and 2, respectively. The curves for each metabolite in the control and in the treated rats were almost parallel and therefore do not suggest a blockade of the metabolites' transport across the blood brain barrier following the administration of ethanol. If the transport of the metabolites were blocked by ethanol then the slopes at any point on the curves for the treated rats will be smaller than those of the controls.

Discussion

The results reported indicate that the effect of ethanol on biogenic amine metabolism is somewhat selective, involving the catecholamines but not the phenolic amines. Thus, while the concentrations of the phenolic amine metabolites (PHPA and PHMA) were not significantly changed by ethanol, those derived from catecholamines (HVA, DOPAC, VMA and MHPG) were significantly increased (see Tables 2 and 3).

The reversal from an oxidative to a reductive pathway prevalent in the metabolism of peripheral biogenic amines was not found to occur in the rat brain for dopamine or noradrenaline. These findings are opposite to observations from human experiments where ethanol reduced the urinary excretion of 5-hydroxyindoleacetic acid (Olson, Gursey & Vester, 1960; Rosenfeld, 1960; Davis *et al.*, 1967a) and VMA (Davis *et al.*, 1967b; Bonham Carter *et al.*, 1970; Ogata *et al.*, 1971) with a concomitant increase of the corresponding alcoholic metabolites, 5-hydroxytryptophol and MHPG. A similar phenomenon was reported in the rat for metabolites of tyramine (Tacker *et al.*, 1970). The above inability of ethanol to produce a reversal in the metabolism of biogenic amines in the brain is supported by reports on the effect of ethanol on 5-hydroxytryptamine metabolism in the whole rat brain (Tyce *et al.*, 1970; Carlsson & Lindqvist, 1973) and in brain slices (Eccleston, Reading & Ritchie, 1969).

The brain metabolism of β-hydroxylated biogenic amines (noradrenaline and octopamine) have been reported by several workers (Rutledge & Jonason, 1967; Breese, Chase & Kopin, 1969a,b; Eccleston *et al.*, 1969) and predicted by a computer simulation approach to produce mostly the alcoholic metabolites (Turner, Illingworth & Tipton, 1974). Biogenic amines with no β-hydroxyl groups, on the other hand, were reported to form mostly the acidic metabolites. The results described here on brain catecholamine metabolites are in agreement with the above observations. Thus, MHPG was found to be the major metabolite of noradrenaline while HVA and DOPAC were found to be the predominant metabolites of dopamine. Furthermore, these metabolites were found to continue to predominate after acute and chronic administration of ethanol (see Table 2 and 3). In this connection, it is interesting to note that although acute ethanol treatment significantly increased the ratio of total MHPG to VMA, chronic ethanol ingestion had no effect on this ratio (see Table 2 and 3).

The observed increase in the concentration of brain catecholamine metabolites following ethanol treatment might result from the blockade of transport or an increased rate of formation; the latter could be due to blockade of catecholamine uptake or to an increased amine release and/or turnover. In order to explain how ethanol influences brain catecholamine metabolism, each of these possibilities warrants consideration.

Inhibition of the transport of dopamine metabolites across the blood brain barrier by ethanol can be excluded on the basis of direct evidence. The data presented here (Figures 1 and 2) illustrate that the rates of depletion of HVA and DOPAC after inhibition of monoamine oxidase by pargyline are not affected by ethanol treatment. However, in a recent report Tabakoff *et al.*, 1975a, claimed that ethanol inhibits 5-hydroxyindoleacetic acid transport from human spinal cord and from the mouse brain (Tabakoff *et al.*, 1975b). If this blockade could be confirmed in the rat brain employing a similar approach to that described here for HVA and DOPAC (see Figures 1 and 2), then these findings will establish for the first time a profound difference in the effect of ethanol on the metabolism of cerebral dopamine and 5-hydroxytryptamine. The failure of ethanol to alter significantly the concentrations of PHPA and PHMA indicates that the transport of these two metabolites is also not affected by ethanol.

Concentrations of ethanol and acetaldehyde within the physiological ranges (Majchrowicz & Mendelson, 1970) were reported to have no effect on the uptake of noradrenaline by synaptic vesicles (Roach, Davis, Pennington & Mordyke, 1973; Lahti & Majchrowicz, 1974). The concentration of ethanol (0.22 M) reported by Israel, Carmichael & MacDonald, 1973, to decrease noradrenaline uptake by synaptic vesicles, is physiologically unattainable. Therefore, at the relatively moderate concentrations of blood ethanol observed in the acutely treated and ethanol-dependent rats, interference with catecholamine uptake cannot be expected to play a very important role in the explanation of the observed increase in brain catecholamine metabolites.

The confused picture on the effect of acute ethanol treatment on brain steady-state levels of catecholamines suggests a complex underlying mechanism that may or may not involve depletion. For example, ethanol was reported by some workers to decrease brain concentration of noradrenaline (Gursey *et al.*, 1959; Gursey & Olson, 1960; Carlsson, Magnusson, Svenson & Waldeck, 1973) while others could not confirm such findings (Murphy, Guze & King, 1962; Effron & Gessa, 1963; Rudas & Vaca, 1964). As for dopamine, the general consensus is that ethanol minimally affects brain dopamine (Haggendal & Lindqvist, 1961; Carlsson *et al.*, 1973; Hunt & Majchrowicz, 1974). No clear opinion, therefore, can be formulated on the possible releasing effect of ethanol *in vivo*. However, *in vitro* evidence suggests that ethanol can release dopamine (Seeman & Lee, 1974; Darden & Hunt, 1975). Whether this release is responsible in whole or part for the observed increase in the catecholamine metabolites following ethanol treatments cannot be ascertained from the present data, and therefore, further experiments are required.

Finally, increased turnover rates of dopamine and noradrenaline are the processes most likely to explain satisfactorily the observed increase (over 50%) in the molar concentrations of their metabolites. Whether the increase in the turnover rates was secondary to catecholamine release or to other phenomena is yet to be resolved. Increased turnover rates of several amines were shown to be associated with increased concentrations of their metabolites (Meek & Neff, 1973; Sharman, 1973). Therefore, the results summarized in Tables 2 and 3, indicate that during the time when ethanol is present in the body, whether after acute or chronic treatments, dopamine and noradrenaline turnover rates are increased. During withdrawal when no ethanol is present in the blood, the dopamine turnover rate either decreases or reverts to normal, while that of noradrenaline remains elevated. The increased turnover of noradrenaline suggested here after ethanol administration is partly supported by reports from several other workers (Corrodi, Fuxe & Hökfelt, 1966; Carlsson *et al.*, 1973; Hunt & Majchrowicz, 1974; Pohorecky, 1974; Pohorecky, Jaffe & Berkeley, 1974). Corrodi *et al.* (1966) could not detect any changes in the turnover of dopamine 15 min after ethanol, while Hunt & Majchrowicz (1974) observed a decrease 2 h after ethanol. Evidence for increased dopamine turnover produced by ethanol was found in two indirect studies. In the first (Carlsson *et al.*, 1973), ethanol was found to increase the production of both radioactive noradrenaline and dopamine from tritiated tyrosine. In the second, moderate doses of ethanol were found to increase the elevation in dihydroxyphenylalanine in both dopamine and noradrenaline dominated areas of the brain following inhibition of aromatic amino acid decarboxylase (Carlsson & Lindqvist, 1973). An increased turnover rate of dopamine following ethanol treatment is indicated from our results after monoamine oxidase inhibition (see Figures 1 and 2). It appears, therefore, that our data are at variance with the reports of Corrodi *et al.* (1966) and Hunt & Majchrowicz (1974) on the effects of ethanol on dopamine turnover. However, we are of the opinion that the turnover rate of dopamine as well as its steady-state levels are dynamically changing after ethanol ingestion. For these reasons, unless stringent precautions are taken to maintain a constant steady-state level of ethanol in the blood as well as sampling the concentration of dopamine at different periods during the course of the experiment, the turnover rates determined may misrepresent what is actually happening. Thus, it is possible that the finding of Corrodi *et al.* (1966) represents the average turnover rate from 15 min after ethanol treatment up to the time the experiment was concluded and that of Hunt & Majchrowicz (1974), represents the average turnover rate 2 h after ethanol administration until the end of the experiment when the blood ethanol levels were falling.

The results reported here allowed us to gain some insight into the mechanism associated with the signs and responses of ethanol withdrawal in the rat. The significant drop in the concentrations of HVA and DOPAC during withdrawal as compared with the intoxicated ethanol-dependent rats (see Table 3) together with the continuous elevation of brain VMA and MHPG clearly indicates that during withdrawal the response of the noradrenergic neurones is different from that of the dopaminergic neurones. The drop in HVA and DOPAC concentrations in the brain indicates that during the withdrawal syndrome, dopamine turnover is lower than in the intoxicated rats, suggesting a decline in dopamine turnover rate following ethanol elimination from the blood. Hunt & Majchrowicz (1974) and Darden & Hunt (1975) observed a reduction in dopamine turnover rate and release during withdrawal. More specific experiments are required to determine whether withdrawal signs and responses are associated with either decreased dopamine and/or increased noradrenaline turnover. These experiments suggest that any stress effects during the withdrawal period are distinct from other stresses (electric shock, cold exposure) which have been shown to elevate dopamine turnover (Bliss & Ailion, 1971).

The authors would like to thank Dr W.A. Hunt for his valuable comments and discussions concerning this manuscript and Ms Sue Stefurak for her skillful technical assistance. Appreciation is extended to Mrs Narcissus Whitley for measuring the blood ethanol, and also to Ms Jane Seaborg for technical assistance.

References

AMSTRONG, M D., SHAW, K N.F. & WALL, P.E. (1956). The phenolic acids of human urine. Paper chromatography of phenolic acid. *J. biol. Chem.*, **218**, 292–303.

BLISS, E.L. & AILION, J. (1971). Relationship of stress and activity to brain dopamine and homovanillic acid. *Life Sci.*, **10**, 1161–1169.

BONHAM CARTER, S., KAROUM, F., SANDLER, M. & YOUDIM, M.B.H. (1970). Promotion of phenolic alcohol formation in man by reserpine and ethanol. *Br. J. Pharmac.*, **39**, 200P.

BREESE, G.R., CHASE, T.N. & KOPIN, I.J. (1969a). Metabolism of tyramine-^{3}H and octopamine-^{3}H by rat brain. *Biochem. Pharmac.*, **18**, 863–869.

BREESE, G.R., CHASE, T.N. & KOPIN, I.J. (1969b). Metabolism of some phenylethylamines and their β-hydroxylated analogs in brain. *J. Pharmac. exp. Ther.*, **165**, 9–13.

CARLSSON, A. & LINDQVIST, M. (1973). Effect of ethanol on the hydroxylation of tyrosine and tryptophan in rat brain *in vivo*. *J. Pharm. Pharmac.*, **25**, 437–440.

CARLSSON, A., MAGNUSSON, T, SVENSON, T.H. & WALDECK, B. (1973). Effect of ethanol on the metabolism of brain catecholamines. *Psychopharmacologia (Berl)*, **30**, 27–36.

CORRODI, H., FUXE, K. & HÖKFELT, T. (1966). The effect of ethanol on the activity of central catecholamine neurons in rat brain. *J. Pharm. Pharmac.*, **18**, 821–823.

DARDEN, J. & HUNT, W A. (1975). Depression of dopamine release during the ethanol withdrawal syndrome. *Pharmacologist*, **17**, Abs. 240.

DAVIS, V.E., BROWN, H., HUFF, J.A. & CASHAW, J.L. (1967a). The alteration of serotonin metabolism to 5-hydroxytryptophol by ethanol ingestion in man. *J. Lab. clin. Med.*, **69**, 132–140.

DAVIS, V.E., BROWN, H., HUFF, J.A. & CASHAW, J.L. (1967b). Ethanol-induced alteration of norepinephrine metabolism in man. *J. Lab. clin. Med.*, **69**, 787–799.

ECCLESTON, D., READING, W.H. & RITCHIE, I.M. (1969). 5-hydroxytryptamine metabolism in brain and liver slices and the effects of ethanol. *J. Neurochem.*, **16**, 274–276.

EFFRON, D.H. & GESSA, G.L. (1963). Failure of ethanol and barbiturates to alter brain monoamine content. *Arch. int. Pharmacodyn.*, **142**, 111.

EWINS, A.J. & LAIDLAW, P.P. (1910). The fate of parahydroxyphenylethylamine in the organism. *J. Physiol. Lond.*, **41**, 78–87.

FELDSTEIN, A. (1971). Effect of ethanol on neurohumoral amine metabolism. In *The Biology of Alcoholism*. Vol. 1. Biochemistry, ed. Kissin, B. & Begleiter, H. pp. 127–259. New York–London: Plenum Press.

FELDSTEIN, A. & SIDEL, C.M. (1969). The effect of ethanol on the *in vivo* conversion of 5-HTP-^{14}C, to serotonin-^{14}C, 5-hydroxyindolealdehyde-^{14}C and its metabolites in rat brain. *Int. J. Neuropharmac.*, **8**, 347–351.

GURSEY, D & OLSON, RE (1960). Depression of serotonin and norepinephrine levels in brain stem of rabbit by ethanol. *Proc. Soc. exp. Biol. Med.*, **104**, 280–286.

GURSEY, D., VESTER, J.W. & OLSON, R.E (1959). Effect of ethanol administration upon serotonin and norepinephrine levels in rabbit brain. *J. clin. Invest.*, **38**, 1008–1009.

HÄGGENDAL, J. & LINDQVIST, M. (1961). Ineffectiveness of ethanol on noradrenaline, dopamine or 5-hydroxytryptamine levels in brain. *Acta pharmac. toxl.*, **18**, 278–280.

HUNT, W A. & MAJCHROWICZ, E. (1974). Alteration in the turnover of brain norepinephrine and dopamine in alcohol-dependent rats. *J. Neurochem.*, **23**, 549–552.

ISRAEL, Y., CARMICHAEL, F.J & MacDONALD, J.A. (1973). Effects of ethanol on norepinephrine uptake and electrically stimulated release in brain tissue. *Ann. N.Y. Acad. Sci.*, **215**, 38–47.

KAROUM, F., LeFEVRE, H, BIGELOW, L B. & COSTA, E. (1973). Urinary excretion of 4-hydroxymethoxyphenylglycol and 4-hydroxy-3-methoxyphenylethanol in man and rat. *Clin. chim. Acta*, **43**, 127–137.

KAROUM, F., GILLIN, J.C. & WYATT, R.J. (1975a). Mass fragmentographic determination of some acidic and alcoholic metabolites of biogenic amines in rat brain. *J. Neurochem.*, **25**, 653–658.

KAROUM, F., GILLIN, J C., WYATT, R.J. & COSTER, E. (1975b). Mass-fragmentography of nanogram quantities of biogenic amine metabolites in human cerebrospinal fluid and whole rat brain. *Biochemical Mass. Spect.*, **2**, 183–189.

LAHTI, R.A (1975). Alcohol, aldehydes and biogenic amines. In *Biochemical Pharmacology of Ethanol*, ed., Majchrowicz, E., pp. 239–253. New York and London: Plenum Press.

LAHTI, R.A. & MAJCHROWICZ, E. (1967). The effects of acetaldehyde on serotonin metabolism. *Life Sci.*, **6**, 1399–1400.

LAHTI, R.A. & MAJCHROWICZ, E. (1969). Acetaldehyde—an inhibitor of the enzymatic oxidation of 5-hydroxyindoleacetaldehyde. *Biochem. Pharmac.*, **18**, 535–538.

LAHTI, R.A. & MAJCHROWICZ, E. (1974). Ethanol and acetaldehyde effects on metabolism and binding of biogenic amines. *Quart. J. Stud. Alc.*, **35**, 1–15.

MAJCHROWICZ, E (1971). Determination of ethanol, methanol and acetone in biological fluids by automated gas chromatography. *Am. Chem. Soc. Div. Biol. Chem.* Abst. 298.

MAJCHROWICZ, E. (1973). Induction of physical dependence on alcohol and the associated metabolic and behavioral changes in rats. *Pharmacologist*, **15**, Abst. 159.

MAJCHROWICZ, E. (1975). Induction of physical dependence upon ethanol and the associated behavioral changes in rats. *Psychopharmacologia (Berl.)*, **43**, 245–254.

MAJCHROWICZ, E. & MENDELSON, J.H. (1970). Blood concentrations of acetaldehyde and ethanol in chronic alcoholics. *Science*, **168**, 1100–1102.

MEEK, J.L. & NEFF, N.H. (1973). The rate of formation of 3-methoxy-4-hydroxyphenylethyleneglycol sulfate in brain as an estimate of the rate of formation of norepinephrine. *J. Pharmac. exp. Ther.*, **184**, 570–575.

MENDELSON, J.H. & LaDOU, J. (1964). Experimentally induced chronic intoxication and withdrawal in alcoholics. Part I: Background and experimental design. *Quart. J. Stud. Alc. Suppl.*, **2**, 1–13.

MURPHY, G.E., GUZE, S.B. & KING, L.J. (1962). Urinary excretion of 5-HIAA in chronic alcoholism. *J.A.M.A.*, **182**, 565.

OGATA, M., MENDELSON, J.H., MELLO, N.K. & MAJCHROWICZ, E. (1971). Adrenal function and alcoholism. II. Catecholamines. *Psychosom. Med.*, **33**, 159–180.

OLSON, R.E., GURSEY, D. & VESTER, J.W. (1960). Evidence for a defect in tryptophan metabolism in chronic alcoholism. *New Eng. J. Med.*, **263**, 1169–1174.

PISANO, J.J., CREVELING, C.R. & UDENFRIEND, S. (1960). Enzymic conversion of p-tyramine to *p*-hydroxyphenylethanolamine (norsynephrine). *Biochim. biophys. Acta*, **43**, 566–569.

POHORECKY, L.A. (1974). Effect of ethanol on central and peripheral noradrenergic neurons. *J. Pharmac. exp. Ther.*, **189**, 380–381.

POHORECKY, L.A., JAFFE, L.S. & BERKELEY, H.A. (1974). Ethanol withdrawal in the rat: Involvement of noradrenergic neurons. *Life Sci.*, **15**, 427–437.

ROACH, M.K. & CREAVEN, P.J. (1968). A micro-method for the determination of acetaldehyde and ethanol in blood. *Clin. chim. Acta*, **21**, 275–278.

ROACH, M.K., DAVIS, D.L., PENNINGTON, W. & NORDYKE, E. (1973). Effect of ethanol on the uptake by rat brain synaptosomes of [^{3}H] DL-norepinephrine, [^{3}H] 5-hydroxytryptamine, [^{3}H] GABA, and [^{3}H] glutamate. *Life Sci.*, **12**, 433–441.

ROSENFELD, G. (1960). Inhibitory influence of ethanol on serotonin metabolism. *Proc. Soc. exp. Biol.*, **103**, 144–149.

RUDAS, N. & VACA, L. (1964). Influenza dell'alcool sul contenuto di monoamine del sistema nervoso centrale. *Acta Neurol. (Napoli)*, **19**, 848–849.

RUTLEDGE, C.O. & JONASON, J. (1967). Metabolic pathways of dopamine and norepinephrine in rabbit brain *in vitro*. *J. Pharmac. exp. Ther.*, **157**, 493–502.

SCHAYER, R.W. (1953). *In vivo* inhibition of monoamine oxidase studied with radioactive tyramine. *Proc. Soc. exp. Biol. Med.*, **84**, 60–63.

SEEMAN, P. & LEE, T. (1974). Dopamine-releasing actions of neuroleptics and ethanol. *J. Pharmac. exp. Ther.*, **190**, 131–140.

SHARMAN, D.F. (1973). The catabolism of catecholamines. *Br. med. Bull.*, **29**, 110–115.

SMITH, A.A. & GITLOW, S. (1967). Effect of disulfiram and ethanol on the catabolism of norepinephrine in man. In *Biochemical Factors in Alcoholism*, ed. Maickel, R.P., pp. 53–99. Oxford: Pergamon Press.

SPECTOR, S., MELMON, K., LOVENBERG, W. & SJOERDSMA, A. (1963). The presence and distribution of tyramine in mammalian tissues. *J. Pharmac. exp. Ther.*, **140**, 229–235.

TABAKOFF, B., BULAT, M. & ANDERSON, R.A. (1975a). Ethanol inhibition of the transport of 5-hydroxyindoleacetic acid from cerebrospinal fluid. *Nature, Lond.*, **254**, 708–710.

TABAKOFF, B., RITZMAN, R.F. & BOGGAN, W.O. (1975b). Inhibition of the transport of 5-hydroxyindoleacetic acid from brain by ethanol. *J. Neurochem.*, *24*, 1043–1051.

TACKER, M., CREAVEN, P.J. & McISSAC, W.M. (1970). Alteration in tyramine metabolism by ethanol. *Biochem. Pharmac.*, **19**, 604–607.

TRUITT, E.B., Jr. & WALSH, M.J. (1971). The role of acetaldehyde in the action of ethanol. In *The Biology of Alcoholism.* Vol. 1: Biochemistry, ed., Kissin, B. & Begleiter, H., pp. 161–195. New York–London: Plenum Press.

TURNER, A.J., ILLINGWORTH, J.A. & TIPTON, K.F. (1974). Simulation of biogenic amine metabolism in the brain. *Biochem. J.*, **144**, 353–360.

TYCE, G.M., FLOCK, E.V., TAYLOR, W.F. & OWEN, C.A., Jr. (1970). Effect of ethanol on 5-hydroxytryptamine turnover in rat brain. *Proc. Soc. exp. Biol.*, **134**, 40–44.

VICTOR, M. & ADAMS, R.D. (1953). The effect of alcohol on the nervous system. *Res. Publ. Ass. nerv. ment. Dis.*, **32**, 526–573.

VON WARTBURG, J.P. (1962). Uptake of 5-hydroxytryptophan-3-^{14}C and its metabolism in rat brain cortex slices. *Can. J. Biochem. Physiol.*, **40**, 1439–1448.

*(Received July 24, 1975.
Revised December 8, 1975.)*

Br. J. Pharmac. (1976), **56**, 413–416

THE UPTAKE OF TRITIATED Δ^1-TETRAHYDROCANNABINOL BY THE ISOLATED VAS DEFERENS OF THE RAT

SANDRA M. EGAN, J.D.P. GRAHAM & M.J. LEWIS
Department of Pharmacology, Welsh National School of Medicine, Heath Park,
Cardrff, CF4 4XN

1 Weighed stripped vasa deferentia were incubated in Holman's solution containing (a) [^{14}C]-sorbitol 0.014 mM, (b) [^{3}H]-noradrenaline ([^{3}H]-NA) 12.31 nM, (c) [^{3}H]-tetrahydrocannabinol ([^{3}H]-Δ^1-THC) 1 µg/ml for 5, 10, 20 and 30 minutes.

2 Tissues were washed, dissolved in Protosol, counted by standard scintillation counting technique and 'drug space' expressed as ct min^{-1} mg^{-1} tissue/ct min^{-1} µl^{-1} bathing fluid.

3 Vasa incubated for 30 min with [^{14}C]-sorbitol were washed for varying lengths of time; 82% clearance had taken place after 2 washes of 5 minutes.

4 The uptake of [^{3}H]-NA was inhibited by the presence of desmethylimipramine (DMI) 10 nM in the bath or by pretreatment of rats with 6-hydroxydopamine (6-OHDA).

5 The uptake of [^{3}H]-Δ^1-THC was not inhibited by the presence of DMI. It was reduced but not abolished by 6-OHDA pretreatment.

Introduction

Δ^1-(*trans*)-Tetrahydrocannabinol (Δ^1-THC) is generally held to be the major psychoactive principal of *Cannabis sativa* L. Previous work has shown that Δ^1-THC suspended in 4% Tween 80 caused hypotension in rats and cats and reduction of the reflex vasoconstriction in the hindlimbs which normally follows the systemic action of a vasodilator drug (Graham & Li, 1973). Analysis of the peripheral activity of Δ^1-THC has so far shown that Δ^1-THC inhibits the uptake of [^{3}H]-noradrenaline ([^{3}H]-NA) into the isolated heart of the rat (Graham, Lewis & Li, 1974a) and that it inhibits in a dose-related manner the release (efflux of ^{3}H) from the isolated vas deferens of the rat which has been equilibrated with [^{3}H]-NA and then electrically stimulated (Graham, Lewis & Li, 1974b). Moreover, if the rat vas was equilibrated with [^{3}H]-Δ^1-THC in Tween 80 and then washed, transmural stimulation released significantly more ^{3}H than during the non-stimulated state and pretreatment of the animals with 6-hydroxydopamine (6-OHDA) abolished this difference. The implication was that Δ^1-THC or a metabolite was released from the adrenergic neurone on appropriate stimulation. In order to complete the evidence about this process studies of the uptake of [^{3}H]-Δ^1-THC into rat vas have been undertaken. We do not extrapolate our findings from vas to brain, even as a hypothesis.

Methods

Uptake studies

Male Wistar rats weighing 100–120 g were killed by a blow on the head. Both vasa were removed and immersed in cold Holman's solution of the following composition (mM) NaCl 120, $NaHCO_3$ 25, NaH_2PO_4 1, KCl 5, $CaCl_2$ 2.5, glucose 11, sucrose 10, ascorbic acid 0.11, disodium edetate 0.04. Each vas was stripped of mesentery, blotted on filter paper and weighed. Pairs of vasa were incubated for 5, 10, 20 and 30 min in 2.5 ml of Holman's solution at 37°C oxygenated with 5% CO_2 in O_2 and containing one of the following: (a) [^{14}C]-sorbitol 0.014 mM in carrier sorbitol to 35.68 mM, (b) [^{3}H]-(–)-NA 12.31 nM in carrier NA to 2.96 µM, (c) [^{3}H]-(–)-NA 12.31 nM in carrier NA to 2.96 µM and 10% v/v ethanol or (d) [^{3}H]-Δ^1-THC 1 µg/ml and 10% v/v ethanol. The concentration of THC is expressed in µg/ml for lack of precise knowledge of the bioavailability of this drug. Incubations for 5–30 min in media (a), (b) and (d) were repeated with the addition of desmethylimipramine (DMI) in a concentration of 10 nM which was found by previous trial to be an optimally effective concentration for preventing the uptake of [^{3}HT-NA in the vas. Finally, the experiment was replicated at the 10 and 30 min incubation times for [^{3}H]-Δ^1-THC and at 30 min for [^{3}H]-NA and [^{14}C]-sorbitol on vasa from rats pretreated with 6-OHDA according to the 14 day schedule of Thoenen & Tranzer (1968).

Sampling and counting

Following incubation, a 100 µl aliquot of the aqueous medium was taken and 10 ml of scintillation fluid containing Triton X-100 added. Vasa were blotted individually, dissolved in 1 ml Protosol solution at 54°C for 18 h and 10 ml scintillation fluid (without Triton X-100) added. All samples were counted by standard technique in an SL 30 liquid Scintillation Counter. The accumulation of each drug was expressed as ct min^{-1} mg^{-1} tissue/ct min^{-1} μl^{-1} medium and termed 'drug space'. Since quenching of samples and efficiency of counting did not vary significantly disintegrations per min were not calculated.

Clearance of [^{14}C]-sorbitol

After incubation of some vasa with [^{14}C]-sorbitol for 30 min the tissues were washed for 5–30 min in 50 ml of gassed Holman's solution at 37°C which was changed every 5 minutes. Based on the findings that 82% of [^{14}C]-sorbitol was cleared by two 5 min washes, this procedure was adopted in all experiments with [^{3}H]-NA and [^{3}H]-Δ^1-THC, in order to remove the majority of the NA and Δ^1-THC from the extracellular space. All results were calculated by ignoring the 18% of the drug remaining within the extracellular space.

Materials

Scintillation fluid: scintillation grade 2; 5-diphenyloxazole 5g; 1,4-di(2-(5-phenyloxazolyl) benzene 0.5 g; toluene 1 litre; Triton X-100. 500 ml was also added for aqueous samples. Protosol; New England Nuclear Chemical Co; desmethylimipramine, Geigy Pharmaceuticals; [^{3}H]-(–)-NA (Radiochemicals, Amersham) specific activity 48 mCi/mg; [^{3}H]-Δ^1-THC (N.I.M.H., D.H.E.W. samples 1480-75-1) specific activity 109 µCi/mg; [^{14}C]-D-sorbitol (Radiochemicals, Amersham) specific activity 9.3 mCi/mg.

Results

Uptake of [^{14}C]-sorbitol

Uptake of [^{14}C]-sorbitol increased with time, saturation occurring between 20 and 30 min (Table 1) where the sorbitol space increases in the time order $5 < 10 < 20$, each value being significantly different from its predecessor. Neither the presence of DMI 10 nM nor pretreatment of rats with 6-OHDA significantly affected the uptake pattern. Two wash periods of 5 min cleared 82% of the sorbitol from the extracellular space.

Uptake of [^{3}H]-noradrenaline

The uptake of [^{3}H]-NA into isolated rat vas is shown in Table 2. Inspection of a best fitting smooth curve applied to these data suggested that there was rapid accumulation during the initial 5 min followed by a slower phase, but saturation had not occurred at 30 minutes. The presence of 10% ethanol did not significantly alter the pattern of NA uptake. DMI 10 nM reduced uptake of [^{3}H]-NA at 30 min only ($P < 0.02$) but was not significantly effective at 5, 10 or 20 minutes. Pretreatment of rats with 6-OHDA also reduced the uptake of [^{3}H]-NA at 30 min ($P < 0.005$), the only time of incubation tested.

Uptake of [^{3}H]-Δ^1-tetrahydrocannabinol

The accumulation curve of [^{3}H]-Δ^1-THC in rat isolated vas is shown in Figure 1. There was an initial rapid uptake followed by a slower phase. The amount of Δ^1-THC bound by the tissue was greater than that of NA at all incubation times. The presence of DMI in a concentration of 10 nM did not alter the accumulation curve. Pretreatment of rats with 6-OHDA

Table 1 Uptake of [^{14}C]-sorbitol by rat vas deferens

Time	*Sorbitol 'space'*	*Sorbitol 'space' in the presence of DMI 10 nM*	*Sorbitol 'space' after pretreatment with 6-OHDA*
5	0.347 ± 0.017 (5)	0.404 ± 0.024 (4)	—
10	0.497 ± 0.024 (5)	0.604 ± 0.470 (5)	—
20	0.848 ± 0.790 (5)	0.709 ± 0.073 (4)	0.794 ± 0.089 (4)

Sorbitol 'space' = ct min^{-1} mg^{-1} tissue/ct min^{-1} μl^{-1} medium Each result is the mean of at least 4 observations (n) ± s.e. mean.
(Student's t test) There was no significant difference in the presence of desmethylimipramine (DMI) 10 nM or after pretreatment with 6-hydroxydopamine (6-OHDA) ($P > 0.2$ in all cases).

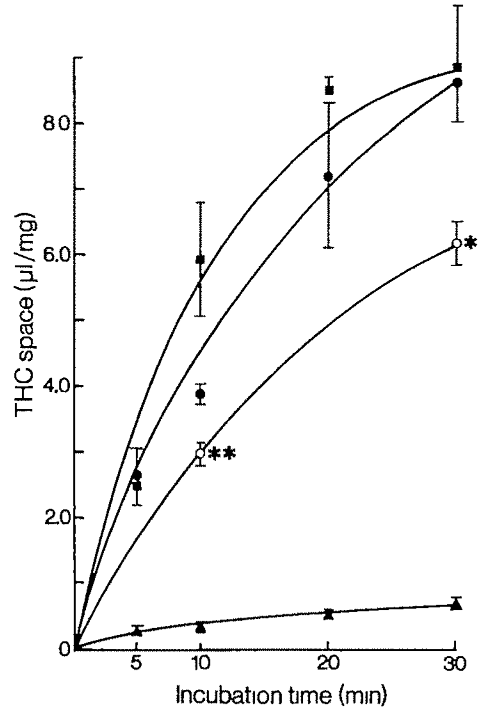


Figure 1 The accumulation of [^{3}H]-Δ^1-tetrahydrocannabinol (THC) in rat vas deferens *in vitro* (●) and the effect of 6-hydroxydopamine (6-OHDA) pretreatment (○) or of desmethylimipramine (DMI) 10 nM (■) on it. For comparison the accumulation of [^{3}H]-(–)-noradrenaline (NA) is also shown (▲). Abscissa scale: time of incubation in minutes. Ordinate scale—'drug space' = ct min^{-1} mg^{-1} tissue/ct min^{-1} µl^{-1} medium. Each result is the mean of at least 4 observations. DMI had no effect, while 6-OHDA reduced the accumulation of Δ^1-THC which was itself much greater than that of NA.
Student's *t* test, * = $P<0.025$; ** = $P<0.005$

significantly reduced accumulation of Δ^1-THC at 10 min ($P<0.005$) and 30 min ($P<0.025$).

Discussion

If D-sorbitol is distributed extracellularly, the estimated values of 70–80% for 'sorbitol space' are large. However, these values were obtained consistently when vasa were incubated with [^{14}C]-sorbitol for 20 and 30 minutes. One explanation for the large extracellular volumes obtained may be that this carbohydrate is distributed to some extent intracellularly as well as extracellularly. This would appear unlikely for two reasons. First, rapid equilibration was obtained with sorbitol (20–30 min) and second, rapid clearance occurred after two 5 min wash periods. The vasa were not cut longitudinally for fear of extensive damage to the muscle and the variable volume of the lumen doubtless accounts for a proportion of the calculated sorbitol space.

The results show that this distribution is not significantly altered by the presence of DMI or pretreatment with 6-OHDA. In contrast, NA is actively accumulated by adrenergic neurones (Iversen, 1967) and this accumulation is significantly reduced by blocking uptake with DMI, or by pretreatment with 6-OHDA, a drug known to destroy adrenergic neurones (Thoenen & Tranzer, 1968).

Since the THC was in alcoholic solution, a control experiment was performed to observe the effect of alcohol on uptake of NA. No significant effect was found. It was assumed that the presence of 10% ethanol was unlikely to affect the accumulation of Δ^1-THC.

Previous work (Graham *et al.*, 1974b) showed that rat vas equilibrated with [^{3}H]-Δ^1-THC released labelled compound following transmural electrical stimulation and that the pretreatment of rats with 6-OHDA abolished this process; it was to be expected

Table 2 Uptake of [^{3}H]-noradrenaline (NA) by rat vas deferens

Time (min)	*NA 'space'*	*NA 'space' in presence of 10% ethanol*	*NA 'space' in presence of DMI 10 nM*	*NA 'space' after pretreatment with 6-OHDA*
5	0.326 ± 0.042 (4)	0.249 ± 0.027 (4)	0.187 ± 0.026 (4)	
10	0.327 ± 0.034 (5)	0.347 ± 0.078 (4)	0.397 ± 0.032 (4)	
20	0.532 ± 0.050 (5)	0.462 ± 0.033 (5)	0.467 ± 0.034 (5)	
30	0.753 ± 0.065 (4)	0.586 ± 0.062 (4)	0.510 ± 0.034 (4)	0.359 ± 0.012 (4)

NA 'space' = ct min^{-1} mg^{-1} tissue/ct min^{-1} µl^{-1} medium. Each result is the mean of at least 4 observations (*n*) ± s.e. mean.
Student's *t* test There was no significant difference in the presence of 10% ethanol Desmethylimipramine (DMI) 10 nM significantly reduced uptake at 30 min ($P<0.02$). Pre-treatment with 6-hydroxydopamine (6-OHDA) also significantly reduced uptake at 30 min ($P<0.005$).

that uptake of [^{3}H]-Δ^1-THC by rat vas should be demonstrable, and be reduced by 6-OHDA. This supposition is confirmed.

This route of accumulation of Δ^1-THC is unlikely to be the same as that for NA because the former was not reduced by DMI. The markedly greater affinity of THC for the tissue relative to NA implies other sites of binding for THC than are available for NA.

We are indebted for [^{3}H]-Δ^1-THC to N.I.D.A. and to the M.R.C. THC was held by one of us (J.D.P.G.) under H.O. licence no. L223.

References

GRAHAM, J.D.P. & LI, D M.F. (1973). Cardiovascular and respiratory effects of Cannabis in rat and cat. *Br. J. Pharmac.*, **49**, 1–10.

GRAHAM, J.D.P , LEWIS, M.J & LI, D.M F. (1974a). The effect of Δ^1-tetrahydrocannabinol on the uptake of [^{3}H]-(–)-noradrenaline by the isolated perfused heart of the rat. *Br. J. Pharmac.*, **51**, 465–466.

GRAHAM, J.D P., LEWIS, M J. & LI, D.M.F (1974b). The effect of Δ^1-tetrahydrocannabinol on the release of [^{3}H]-(–)-noradrenaline from the isolated vas deferens of the rat. *Br. J. Pharmac.*, **52**, 233–236.

IVERSEN, L.L. (1967). *The uptake and storage of noradrenaline in sympathetic nerves.* Cambridge University Press.

THOENEN, H. & TRANZER, J.P. (1968). Chemical sympathectomy by selective destruction of adrenergic nerve endings with 6-hydroxydopamine. *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.*, **261**, 271–288.

*(Received July 30, 1975.
Revised November 26, 1975.)*

Br. J. Pharmac. (1976), **56**, 417–421

UPTAKE OF 5-HYDROXYTRYPTAMINE BY RAT BLOOD PLATELETS AND ITS INHIBITION BY ADENOSINE 5′-DIPHOSPHATE

A.H. DRUMMOND & J.L. GORDON

Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP

1 The uptake of 5-hydroxytryptamine (5-HT) by rat blood platelets in citrated plasma was linear for at most 10 s and was substantially complete within 3 minutes.

2 Adenosine 5′-diphosphate (ADP) was a potent inhibitor of 5-HT uptake ($K_I = 0.38\ \mu M$) and kinetic analysis revealed that the inhibition was not competitive.

3 Inhibition of 5-HT uptake by ADP was abolished in the presence of prostaglandin E_1 and 2-*n*-amylthio-AMP, which also inhibit the stimulant actions of ADP on blood platelets.

4 It is concluded that ADP could inhibit 5-HT uptake by changing the Na^+/K^+ distribution across the cell membrane, and the biological significance of this is discussed.

Introduction

Adenosine 5′-diphosphate (ADP) and 5-hydroxytryptamine (5-HT) exert stimulatory effects on blood platelets, that is, they cause the cells to change in shape and, under appropriate conditions, to form aggregates (Gaarder, Jonsen, Laland, Hellem & Owren, 1961; Mitchell & Sharp, 1964). These agents exert their effects by interacting with specific recognition sites on the cell membrane (Born, 1965; Baumgartner & Born, 1968; Nachman & Ferris, 1974; Drummond & Gordon, 1975). Platelets also contain a highly efficient uptake process for 5-HT (for review see Sneddon, 1973), and this would be expected to reduce the stimulatory effect of the amine.

Both ADP and 5-HT are stored in platelet intracellular organelles and are released in parallel when platelets are stimulated by agents such as collagen or thrombin (Holmsen, Day & Stormorken, 1969). Since the growth of a haemostatic plug is promoted by the stimulatory effects of extracellular ADP and 5-HT, the interaction of these agents at the platelet membrane is of some biological significance. We have found that ADP is a potent inhibitor of 5-HT uptake by rat blood platelets and suggest that this interaction could contribute to the efficiency of the haemostatic process.

Methods

Preparation of platelet-rich plasma (PRP)

PRP was prepared from citrated rat blood as described previously (Gordon & Drummond, 1974). Platelet counts in PRP ranged from 0.8 to $1{\cdot}2 \times 10^9$ cells/ml.

Uptake of 5-hydroxy [^3H] tryptamine by platelets

Uptake of 5-HT was measured by incubating 0.1 ml samples of PRP at 37°C with 0.2 to 2 μM 5-hydroxy [^3H] tryptamine ([^3H]-5-HT). PRP samples were preincubated for 60 s at 37°C before adding [^3H]-5-HT in a volume of 5 μl and briefly vortex-mixing. After 10 s incubation with [^3H]-5-HT, 0.5 ml volumes of ice-cold iso-osmotic saline containing 0.4% w/v disodium edetate (EDTA) were added and samples centrifuged for 30 s at 14,700 *g* in a Quickfit microcentrifuge; control experiments showed that the ice-cold EDTA-saline halted 5-HT uptake. The supernatants were decanted and the platelet pellets washed once with 1.0 ml volumes of ice-cold EDTA-saline; the amount of ^3H removed by washing was comparable with that in control experiments using samples of cell-free plasma. The pellets were then digested by incubation at 80°C for 10 min with 0.5 ml volumes of 19 M formic acid, and transferred to vials containing 10 ml of scintillant (toluene with 0.33% w/v 5-(4-biphenylyl)-2-(4-*t*-butylphenyl)-1-oxa,3,4 diazole (butyl PBD) plus 30% (v/v ethoxyethanol). Radioactivity was measured in a Nuclear Chicago Mk. 2 liquid scintillation counter. In some experiments uptake of [^3H]-5-HT was measured as the decrease in supernatant radioactivity after separation of platelets and plasma by centrifugation (see legend of Figure 1).

Measurement of endogenous 5-hydroxytryptamine

Endogenous 5-HT in platelets and plasma was measured fluorimetrically after condensation with *o*-phthaldialdehyde as previously described (Drummond & Gordon, 1974).

Materials

5-Hydroxy [G-^{3}H] tryptamine creatinine sulphate (0.5 Ci/mmol), from the Radiochemical Centre, Amersham, Bucks., was dissolved in 0.2 M sodium acetate-acetic acid buffer, pH 5.5, to a concentration of 10^{-4} M and stored in 0.5 ml volumes at −20°C. It was diluted before use to the required concentration with 0.5 M Tris(hydroxymethyl)aminomethane buffer, pH 7.4. ADP and EDTA, from the Sigma Chemical Co., Kingston-upon-Thames, Surrey were dissolved in iso-osmotic saline. Prostaglandin E_1 (Upjohn Co., Kalamazoo, Michigan, U.S.A.) was dissolved and stored as described previously (MacIntyre & Gordon, 1974). Indomethacin (Merck, Sharp & Dohme, Hoddesdon, Herts) was dissolved in 0.5% w/v sodium carbonate (pH 10.3). The pH of the solution was adjusted to 7.5 by addition of 1 N HCl and further dilutions were made with iso-osmotic saline. 2-*n*-Amylthio AMP (Kohjin Co. Ltd., Saiki, Oita, Japan) was dissolved in iso-osmotic saline.

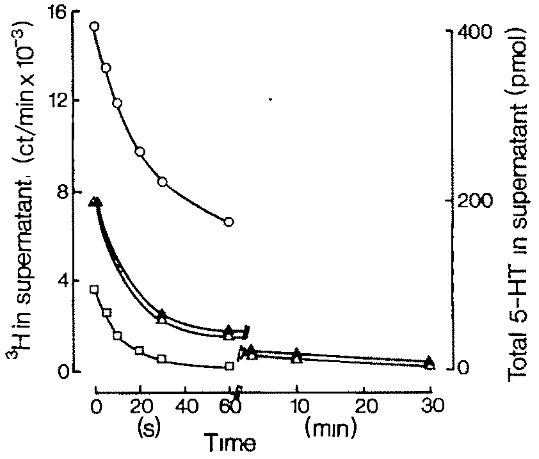


Figure 1 Rate of uptake of 5-hydroxytryptamine (5-HT) by rat blood platelets in citrated platelet-rich plasma (PRP). PRP (0.2 ml) was pre-incubated at 37°C for 1 min before the addition of [^{3}H]-5-HT At each time-point 0.5 ml of ice-cold 0.4% w/v EDTA in saline was added and samples were spun at 14,700 *g* for 30 seconds Samples (0.25 ml) of the supernatant were taken for total 5-HT assay (closed symbols) and 0.1 ml samples were taken for the measurement of radioactivity (open symbols) as described in the methods section. Initial [^{3}H]-5-HT concentrations added were: (○) 2 μM, (△) 1 μM, (□) 0.5 μM. Each point is the mean of duplicate determinations

Results

Uptake of 5-hydroxy [^{3}H]-tryptamine

Using substrate concentrations of 0.5 to 2.0 μM, uptake of [^{3}H]-5-HT was approximately linear for 10 s and was largely completed within 3 min (Figure 1). To determine whether any exchange occurred between endogenous platelet 5-HT and added [^{3}H]-5-HT during the uptake process, the total 5-HT in the cell-free supernatant was measured fluorimetrically over a 30 min period in aliquots of the samples taken for radioactivity measurements. Changes in total 5-HT paralleled the changes in radioactivity, indicating that the uptake process was not accompanied by measurable 5-HT exchange (Figure 1). Lineweaver-Burk analysis revealed that the uptake process was saturable; the K_m value for 5-HT uptake by rat blood platelets was 0.83 ± 0.29 μM (mean ± s.e.; $n = 9$) and the V_{max} was 63.2 ± 23.9 pmol/10^8 cells per 10 seconds. These values were derived from 9 separate experiments, and the results of one experiment using a small range of 5-HT concentrations are shown in Figure 2.

Inhibition of uptake by adenosine 5′-diphosphate

When PRP samples were pre-incubated for 60 s at 37°C with ADP (0.4 to 3.3 μM) before the addition of [^{3}H]-5-HT, uptake was inhibited. The pattern of inhibition was not competitive (Figure 2). Analysis of the data in Figure 2 by the method of Plowman (1972) revealed that the K_i for ADP was 0.38 μM. Inhibition of [^{3}H]-5-HT uptake by ADP was short-lived. When PRP samples were pre-incubated with ADP, inhibition of 5-HT uptake decreased with increasing time of pre-incubation. In the presence of 1.66 μM ADP uptake had returned to control levels after 10 min; using 6.6 μM, 5-HT uptake was inhibited by 85% after 1 min pre-incubation with ADP, 53.6% inhibited after 10 min and 34.4% inhibited after 20 min (Figure 3).

Modification of the inhibitory effect of adenosine 5′-diphosphate by drugs

The observed inhibition of [^{3}H]-5-HT uptake by ADP could conceivably have resulted from ADP releasing endogenous platelet 5-HT, but when rat platelets were exposed to ADP in the concentration range used for these experiments we could detect no release of platelet 5-HT.

In support of this conclusion, incubation of PRP samples with drugs which inhibit the release of platelet constituents (e.g. 0.1 mM indomethacin) did not affect the inhibition of [^{3}H]-5-HT uptake by ADP. However, agents which inhibit the direct platelet-stimulating action of ADP (0.6 μM prostaglandin E_1; 20 μM 2-*n*-amylthio AMP) abolished the inhibition of [^{3}H]-5-HT uptake by ADP (Table 1). 2-*n*Amylthio AMP (0.1 mM) and prostaglandin E_1 (0.6 μM) had no direct effect on the uptake of [^{3}H]-5-HT.

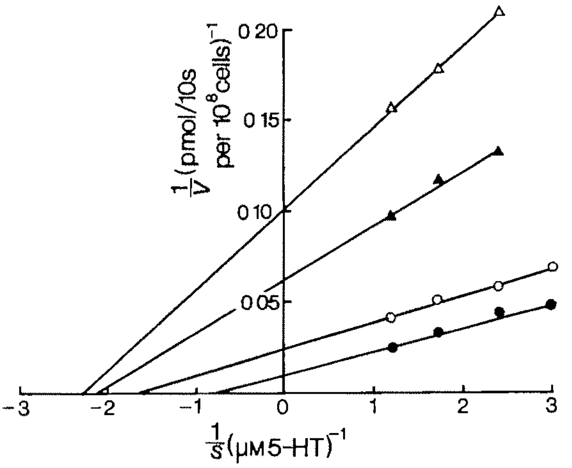


Figure 2 Double reciprocal analysis of the effect of adenosine 5′-diphosphate (ADP) on 5-hydroxytryptamine (5-HT) uptake by rat platelets. Initial velocity (V) is expressed as [^{3}H]-5-HT uptake in units of pmol/10 s per 10^8 cells. [^{3}H]-5-HT concentrations used in the experiment shown were in the range 0.3 to 0.8 µM, but in other experiments concentrations up to 2 µM were used. Additions were: (●) saline; (○) 0.415 µM ADP; (▲) 1.66 µM ADP; (△) 3.32 µM ADP. Lines were constructed by linear regression analysis, and each point is the mean of duplicate determinations.

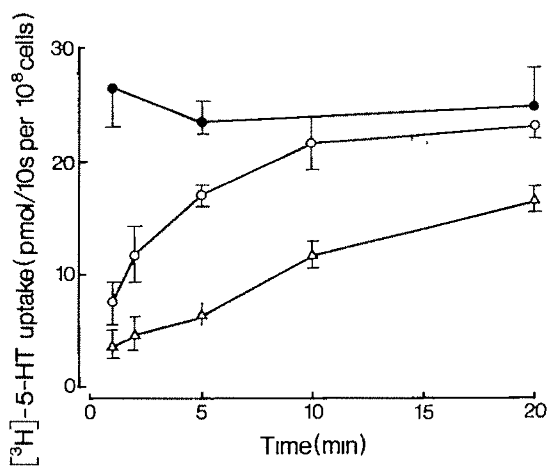


Figure 3 Adenosine 5′-diphosphate (ADP) inhibition of 5-hydroxytryptamine (5-HT) uptake by rat blood platelets: effect of pre-incubating ADP in platelet-rich plasma (PRP). PRP was pre-incubated at 37°C with: saline (●); 1 6 µM ADP (○); or 6.6 µM ADP (△); for 1, 2, 5, 10 or 20 min as indicated before addition of 0.415 µM [^{3}H]-5-HT. Uptake was measured as described in the text. Each point is the mean of triplicate determinations, and the vertical bars show the range of values obtained (in three cases, only one half of the range is indicated).

Table 1 Adenosine 5′-diphosphate (ADP) inhibition of 5-hydroxytryptamine (5-HT) uptake by rat blood platelets: effect of drugs

Compound (µM)	*% inhibition of 5-HT uptake by ADP*
Saline	96
2-*n*-Amylthio AMP (1)	95
2-*n*-Amylthio AMP (10)	46
2-*n*-Amylthio AMP (20)	8
Prostaglandin (0.03)	93
Prostaglandin (0.15)	59
Prostaglandin (0.60)	16
Indomethacin (10)	96
Indomethacin (30)	94
Indomethacin (100)	97

PRP (0.1 ml) was pre-incubated at 37°C for 1 min with drugs or an equivalent volume of isotonic saline prior to the addition of 10 µM ADP or saline. One min later, 0 6 µM [^{3}H]-5-HT was added and the uptake was terminated after 10 s by the addition of EDTA-saline. Uptake was measured as described in the text. Each value is the mean of triplicate determinations on PRP samples from at least two rats.

Discussion

The uptake of submicromolar concentrations of [^{3}H]-5-HT by rat blood platelets in citrated plasma was largely complete within 1 min, with a linear uptake component which lasted, at most, 10 seconds. These findings are in close agreement with the results of Buczko, de Gaetano & Garattini (1975), who studied uptake of [^{14}C]-5-HT by rat platelets in citrated plasma. Sneddon (1969), using rat platelets washed free of plasma and suspended in a protein-free salt solution, reported that uptake of [^{3}H]-5-HT was linear for at least 12 min, but analysis of his data reveals that the rate of 5-HT uptake was much lower in his experiments than in ours. The reason for this may be that the platelets were damaged during the washing procedure or that the medium in which they were finally suspended lacked some plasma component essential for the efficient function of the 5-HT uptake process.

Although the results of the present study reveal a hitherto unrecognized action of ADP on blood platelets, which could be of some biological significance, the mechanism of action remains unclear.

5-HT uptake by blood platelets is an active process obeying Michaelis-Menten kinetics which apparently utilizes the energy inherent in the spontaneous movement of Na^+ and K^+ across the platelet membrane. Platelets have low sodium and high potassium levels relative to plasma and if this asymmetric ion distribution is not maintained the 5-HT uptake process does not function efficiently (Sneddon, 1969; 1971). The uptake of 5-HT by platelets can be inhibited by agents which combine directly with the uptake site, such as chlorimipramine and other tricyclic anti-depressants. Kinetic studies of the effect of these compounds have invariably shown the inhibition to be competitive in nature (Stacey, 1961; Yates, Todrick & Tait, 1964; Tuomisto, 1974) and we can therefore exclude the possibility that ADP is acting in this manner. Uptake can be non-competitively inhibited by agents which block glycolysis (Hughes & Brodie, 1959; Weissbach & Redfield, 1960; Sneddon, 1971; Campbell & Todrick, 1973) or by inhibitors of $(Na^+ + K^+)$-ATP-ases, such as ouabain (Weissbach, Redfield & Titus, 1960; Sneddon, 1971; Campbell & Todrick, 1973). Both these types of inhibitor appear to act by interfering with the asymmetric ion distribution across the membrane which is necessary for efficient 5-HT uptake (Tissari, Schönhöfer, Bogdanski & Brodie, 1969; Sneddon, 1971).

At physiological pH values ADP is an impermeant anion, and available evidence indicates that its stimulant effects on platelets are mediated by specific interaction with sites on the exterior of the cell membrane (Nachman & Ferris, 1974). The rapid onset of ADP's inhibitory effect on 5-HT uptake is consistent with a similar mode of action, and the reduced potency of ADP when preincubated in PRP could be the result of its degradation by plasma enzymes. Moake, Ahmed, Bachur & Gutfreund (1970) reported that ADP was a potent non-competitive inhibitor of platelet membrane ATP-ase activity, and suggested that this might mediate its stimulant action. Inhibition of the ATP-ase should alter the distribution of Na^+ and K^+ across the platelet membrane, and indeed Feinberg, Scorer, Lebreton, Grossman & Born (1975) found that ^{22}Na uptake by platelets was rapidly and transiently stimulated by 10 µM ADP. It therefore seems possible that the stimulant effects of ADP and its inhibition of 5-HT uptake may both be linked to a change in transmembrane ionic distribution.

In support of the concept that these actions of ADP share a common mechanism are our findings that inhibition of 5-HT uptake is blocked by prostaglandin E_1 and 2-*n*-amylthio AMP, at concentrations which also inhibit the platelet shape change induced by ADP. Furthermore, the concentration of ADP (0.1 to 0.2 µM) which induces a half-maximal platelet shape change in rat citrated PRP is close to its K_i value (0.38 µM) for inhibition of 5-HT uptake (Drummond & Gordon, unpublished observations). It is also conceivable that ADP might inhibit 5-HT uptake simply by inducing the platelets to change in shape, if this resulted in masking of uptake sites on the cell membrane. This seems most unlikely, however, because other agents (e.g. 5-methoxy-α-methyltryptamine) can induce a shape change in rat platelets without affecting 5-HT uptake (Drummond & Gordon, 1975). We can therefore conclude that inhibition of 5-HT uptake is a specific property of ADP, and not a general effect associated with the shape change.

Although further work is necessary to establish the mechanisms by which ADP inhibits 5-HT uptake, the immediate biological significance of this effect is evident: maintaining a higher extracellular 5-HT level helps promote the growth of platelet aggregates and can thus increase haemostatic efficiency. Of greater potential significance, however, is the possibility that this effect may not be restricted to platelets: the transmembrane Na^+/K^+ distribution regulates amine uptake in the central nervous system (CNS) as well as in platelets (Bogdanski, Tissari & Brodie, 1968; Tissari *et al.*, 1969) and stimulating Na^+ influx by batrachotoxin or veratridine inhibits dopamine uptake by rat striatal synaptosomes (Holz & Coyle, 1974). Since adenine nucleotides are released in conjunction with amine neurotransmitters, it would seem important to establish whether ADP can stimulate Na^+ influx in the CNS as well as in platelets (Feinberg *et al.*, 1975), and, if so, whether this provides a basis for control of transmitter re-uptake.

A.H.D. is an M.R.C. scholar. We thank Dr J.E. Pike of Upjohn Co., Kalamazoo, Mich., U.S.A. for donating prostaglandin E_1 and Dr K. Kikugawa of Kohjin Co., Saiki, Oita, Japan for donating 2-*n*-amylthio AMP.

References

BAUMGARTNER, H.R. & BORN, G.V.R. (1968). Effects of 5-hydroxytryptamine on platelet aggregation. *Nature, Lond.*, **218**, 137–141.

BOGDANSKI, D.F., TISSARI, A. & BRODIE, B.B. (1968). Role of sodium, potassium, ouabain and reserpine in uptake, storage and metabolism of biogenic amines in synaptosomes. *Life Sci.*, **7**, 419–428.

BORN, G.V.R. (1965). Uptake of adenosine and of adenosine diphosphate by human blood platelets. *Nature, Lond.*, **206**, 1121–1122.

BUCZKO, W, de GAETANO, G. & GARATTINI, S. (1975). Effect of fenfluramine on 5-hydroxytryptamine uptake and release by rat blood platelets. *Br. J. Pharmac.*, **53**, 563–568.

CAMPBELL, I C & TODRICK, A (1973). On the pharmacology of the amine-uptake mechanism in human blood platelets. *Br. J. Pharmac.*, **49**, 279–287.

DRUMMOND, A.H. & GORDON, J.L. (1974). Rapid, sensitive microassay for platelet 5-HT. *Thromb. Diath. Haemorrh.*, **31**, 366–367.

DRUMMOND, A.H. & GORDON, J.L. (1975). Specific binding sites for 5-hydroxytryptamine on rat blood platelets. *Biochem. J.*, **150**, 129–132.

FEINBERG, H , SCORER, M., LEBRETON, G., GROSSMAN, B , & BORN, G V.R. (1975). Adenosine diphosphate-induced ^{22}Na uptake by human blood platelets. *Circulation*, **50**, 1059A.

GAARDER, A., JONSEN, J., LALAND, S., HELLEM, A. & OWREN, P.A (1961). Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature, Lond.*, **192**, 531–532.

GORDON, J.L. & DRUMMOND, A.H. (1974). A simple fluorimetric microassay for adenine compounds in platelets and in plasma, and its application to studies on the platelet release reaction. *Biochem. J.*, **138**, 165–169.

HOLMSEN, H., DAY, H.J. & STORMORKEN, H. (1969). The blood platelet release reaction. *Scand. J. Haemat.*, suppl. **8**, 1–26.

HOLZ, R.W. & COYLE, J.T. (1974). The effects of various salts, temperature, and the alkaloids veratridine and batrachotoxin on the uptake of [^{3}H]-dopamine into synaptosomes from rat striatum. *Mol. Pharmac.*, **10**, 746–758.

HUGHES, F B & BRODIE, B.B (1959). The mechanism of serotonin and catecholamine uptake by platelets. *J. Pharmac. exp. Ther.*, **127**, 96–102.

MACINTYRE, D.E. & GORDON, J L. (1974). The prostaglandin receptor on blood platelets: effect of sulphydryl reagents on inhibition of platelet aggregation by PGE$_1$. *Biochem. Soc. Transactions*, **2**, 1265–1269.

MITCHELL, J.R.A. & SHARP, A.A. (1964). Platelet clumping in vitro. *Br. J. Haemat.*, **10**, 78–93.

MOAKE, J.L., AHMED, K., BACHUR, N.R. & GUTFREUND, D.E (1970). Mg^{2+}-dependent, (Na$^+$ + K$^+$)-stimulated ATPase of human platelets: properties and inhibition by ADP. *Biochim. Biophys. Acta.*, **211**, 337–344.

NACHMAN, R.L. & FERRIS, B. (1974). Binding of adenosine diphosphate by isolated membranes of human platelets. *J. biol. Chem.*, **249**, 704–710.

PLOWMAN, K.M. (1972). *Enzyme Kinetics*, p.61, New York: McGraw Hill.

SNEDDON, J M (1969). Sodium-dependent accumulation of 5-hydroxytryptamine by rat blood platelets. *Br. J. Pharmac.*, **37**, 680–688.

SNEDDON, J.M (1971). Relationship between internal Na$^+$/K$^+$ and the accumulation of [^{14}C]-5-hydroxytryptamine by rat platelets. *Br. J. Pharmac.*, **43**, 834–844.

SNEDDON, J.M. (1973). Blood platelets as a model for monoamine-containing neurones. In *Progress in Neurobiology* ed Kerkut, G.A. & Phillis, J.W., Vol., 1, pp.151–198. Oxford: Pergamon Press.

STACEY, R.S. (1961). Uptake of 5-hydroxytryptamine by platelets. *Br. J. Pharmac. Chemother.*, **16**, 284–295.

TISSARI, A H., SCHÖNHOFER, P.S., BOGDANSKI, D.F. & BRODIE, B.B. (1969). Mechanism of biogenic amine transport. Relationship between sodium and the mechanism of ouabain blockade of the accumulation of serotonin and norepinephrine by synaptosomes. *Mol. Pharmac.*, **5**, 593–604.

TUOMISTO, J. (1974). A new modification for studying 5HT uptake by blood platelets: a re-evaluation of tricyclic antidepressants as uptake inhibitors. *J. Pharm. Pharmac.*, **26**, 92–100.

WEISSBACH, H. & REDFIELD, B.G. (1960). Factors affecting the uptake of 5-hydroxytryptamine by human platelets in an organic medium. *J. biol. Chem.*, **235**, 3287–3291.

WEISSBACH, H., REDFIELD, B.G. & TITUS, E.O (1960). The effect of cardiac glycosides and inorganic ions on binding of serotonin by platelets. *Nature, Lond.*, **185**, 99–100.

YATES, C M , TODRICK, A. & TAIT, A.C (1964). Effect of imipramine and some analogues on the uptake of 5-hydroxytryptamine by human blood platelets in vitro. *J. Pharm. Pharmac.*, **116**, 46–463.

(Received August 4, 1975
Revised November 27, 1975.)

Br. J. Pharmac. (1976), **56**, 423–429

THE DISTRIBUTION AND KINETICS OF SULPHACETAMIDE IN LEUKAEMIC MICE

C. MARCHAND & D. NADEAU

Département de pharmacologie, Faculté de médecine, Université de Montréal,
Montréal, Québec, Canada H3C 3J7

1 During the first 90 min following oral administration of sulphacetamide, there was a rapid decline in plasma drug concentration in control mice whereas a progressive increase in sulphacetamide concentration was observed in leukaemic mice.

2 Similar changes in the kinetics of sulphacetamide distribution were observed in the liver, spleen and muscle.

3 While the concentration of sulphacetamide remained quite constant in the brain and fat tissue of control mice, a progressive increase in drug concentration was observed in the brain and fat tissue of leukaemic mice.

4 Some of these changes in the kinetics of sulphacetamide tissue distribution are compatible with delay in gastrointestinal absorption of the drug and its accumulation in the ascitic fluid.

Introduction

It is well established that there is a decrease in drug metabolizing enzyme activity in the liver of tumour-bearing rats (Adamson & Fouts, 1961; Kato, Frontino & Vassanelli, 1963). This is associated with prolongation of drug action when it is administered by the intravenous or intraperitoneal route (Kato, Takanaka & Oshima, 1968; Rosso, Donelli, Franchi & Garattini, 1971).

In recent studies, it was shown that not only drug biotransformation was affected by the disease but tissue distribution kinetics of sulphacetamide were modified in Walker tumour-bearing rats (Nadeau & Marchand, 1975). These changes could be partially explained by delay in gastrointestinal absorption of the drug. Decrease in the rate of drug elimination was also believed to play a role in these changes of distribution kinetics. Since these observations were made with a solid type of tumour, it was of interest to study the effect of leukaemia L-1210 on the distribution of sulphacetamide in another species, the mouse.

Methods

BDF[1] male mice weighing between 18 and 20 g were obtained from Sprague-Dawley, Madison, Wisconsin. The leukaemia strain was obtained through the courtesy of Microbiological Ass., Bethesda, Maryland. The ascitic fluid was aspirated with sterile instruments from the abdominal cavity of acutely ill mice and 0.1 ml of this fluid, containing 10^6 to 10^7 leukocytes was injected into the peritoneal cavity of healthy mice. The survival time of these animals was 6 to 7 days.

Five days after injection of ascitic fluid, animals were starved overnight but allowed water *ad libitum.* Their weights were 18.9 ± 0.3 g and 23.1 ± 0.5 g for control and leukaemic mice respectively. These mice were then given sulphacetamide sodium 200 mg/kg, by the oral route in a volume of 20 ml/kg. At different times after drug administration, the mice were killed by decapitation and the blood collected in heparin-treated tubes. The abdominal cavity was then opened and the ascitic fluid aspirated with a syringe. After ligature of the cardia and the pylorus, the stomach and the intestine were taken out as well as the liver, spleen, kidney, brain, fat tissue (50–400 mg) from the epididymal region, and 250 to 450 mg of striated muscle from the hind legs.

The tissue was then homogenized (Polytron 10) in a 5% solution of trichloroacetic acid. Determination of sulphacetamide was made on clear supernatants after centrifugation at 2,500 rev/min for 10 minutes. Whenever the supernatant was not clear, fat was extracted with chloroform. Determination of sulphacetamide was made by the method of Bratton & Marshall (1939), as modified by Way, Smith, Howie, Weiss & Swanson (1948). Since tissue blanks gave a slight but detectable colouration at 545 nm, readings were corrected with blanks prepared from tissues of control and leukaemic mice.

Plasma half-life ($T_{1/2}$) of sulphacetamide was approximated from the regression line obtained from the log of drug plasma concentration *vs* time. This regression line was calculated from individual data and the correlation coefficient (r) estimated. The error variance, the 95% confidence limits for the regression line and the 95% confidence intervals of the slope (β) were calculated as described by Goldstein (1964).

The areas under the curves (AUC) were approximated as sums of trapezoids. Let C_i and C_i' be the mean sulphacetamide concentration in the plasma at time t_i of a group of control mice and of a group of leukaemic animals respectively. Let A and A' be the area under the curve for control and for leukaemic mice respectively. Then, for the control group,

$$\begin{aligned} A &= (t_2-t_1)(C_1+C_2)/2 + (t_3-t_2) \\ &\quad (C_2+C_3)/2 + (t_4-t_3)(C_3+C_4)/2 \\ &\quad + (t_5-t_4)(C_4+C_5)/2 \end{aligned}$$

which may be written in the form

$$A = \sum_{i=1}^{5} k_i C_i$$

$$\begin{aligned} \text{where } k_1 &= (t_2-t_1)/2 \\ k_i &= (t_{i+1}-t_{i-1})/2 \\ &\quad \text{for} \quad i = 2, 3, 4; \quad \text{and} \\ k_5 &= (t_5-t_4)/2 \end{aligned}$$

Replacing C_i by C_i' yields A'.

The difference between the two areas thus becomes

$$\bar{D} = A - A' = \sum_{i=1}^{5} k_i (C_i - C_i')$$

To test the null hypothesis between the two areas one may use the statistic

$$t = \bar{D}/S_{\bar{D}}$$

where

$$S^2_{\bar{D}} = \sum_{i=1}^{5} k_i^2 (S_i^2 + S_i'^2), \quad S_i^2 \quad \text{and} \quad S_i'^2$$

being the estimates of the variances of the means for each group at time t_i. On the assumption that the mean concentrations follow normal distributions with different variances, Welch (1947) showed that the distribution of t may be approximated by Student's t-distribution with f degrees of freedom, where

$$f = \frac{\left[\sum_{i=1}^{5} k_i^2 (S_i^2 + S_i'^2)\right]^2}{\sum_{i=1}^{5} \frac{(k_i^2)^2 (S_i^2)^2}{n_i + 1} + \sum_{i=1}^{5} \frac{(k_i^2)^2 (S_i'^2)^2}{n_i' + 1}} - 2$$

n_i and n_i' being the number of mice in each group at time t_i in the control and cancerous group respectively.

Significance of the difference between control and leukaemic mice was assessed by the t-test and a P value of 0.05 or less was considered significant.

Results

As illustrated in Figure 1, peak sulphacetamide concentrations were reached within 10 min after drug administration in control mice. The drug was rapidly cleared from the blood as indicated by the relatively short half-life of the drug (Figure 2). In leukaemic mice, a very different pattern was found (Figure 1); peak drug concentration was reached 90 min after drug administration.

In the liver as well as in the spleen (Figure 1), the kinetics of sulphacetamide distribution was similar to that observed in the plasma for both control and leukaemic animals. The concentration of muscle sulphacetamide as a function of time was not markedly different from that of the above tissues except that peak drug concentration was not reached until 20 min after its administration (Figure 3). In leukaemic mice, there was a gradual increase in drug concentration as observed in other tissues. Sulphacetamide concentrations in the brain of control animals remained quite low and relatively constant throughout the observation periods (Figure 3). This is in contrast with leukaemic mice where, in the early periods of observation, lower brain concentrations of sulphacetamide were followed by constantly higher concentrations than in control animals; concentrations of the drug as a function of time are not unlike those observed in the other tissues of leukaemic mice. Except at the 20 min period, drug concentration in the fat tissue remained fairly constant in control animals (Figure 3). However, in leukaemic mice, there was progressive increase in the concentration of sulphacetamide in the tissue to reach a plateau 90 min after its administration.

Since up to 3.0 ml of ascitic fluid was recovered from leukaemic mice, it was important to measure the drug in that 'compartment'. Sulphacetamide concentrations in ascitic fluid were compared to those found in the plasma (Figure 4). Except for the 120 min period, sulphacetamide concentrations were found to be higher in the plasma than in the ascitic fluid.

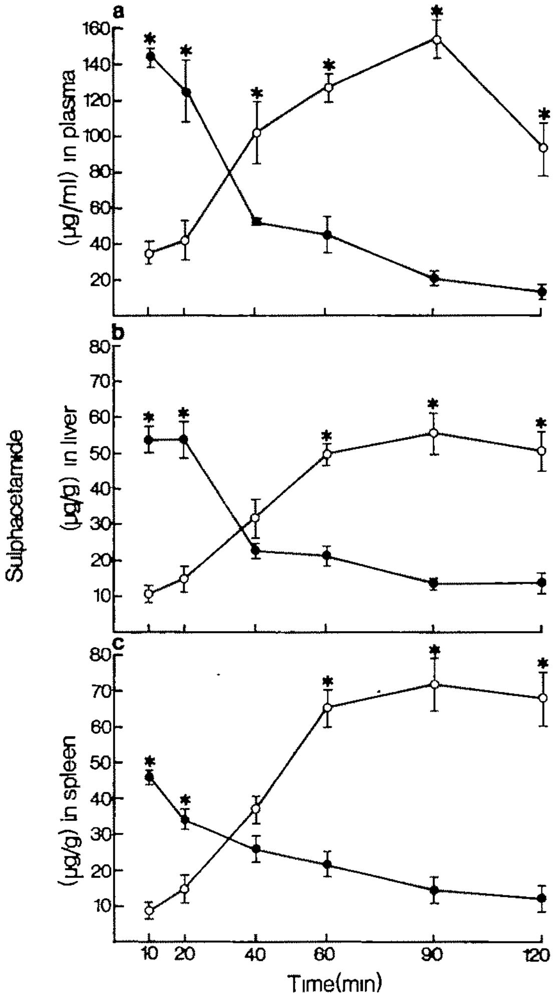


Figure 1 Sulphacetamide concentration in (a) plasma, (b) liver and (c) spleen at different times after oral administration of sulphacetamide sodium, 200 mg/kg in control (●) and leukaemic mice (L-1210) (○), 5 days after inoculation. Each point represents the mean results from 5 to 6 mice. Vertical lines show standard error. *$P<0.05$.

Expressed in terms of percentage of the administered dose, sulphacetamide accumulation became important 40 min after its administration and reached a plateau at the 60 min period, with close to 5% of the administered dose recovered in the ascitic fluid.

As shown in Figure 5, sulphacetamide equilibrium between tissue and plasma was slow to be reached in control as well as in leukaemic mice. In all tissues of leukaemic animals, the tissue/plasma concentration ratios were found to be lower than those of control mice in the last periods of observation.

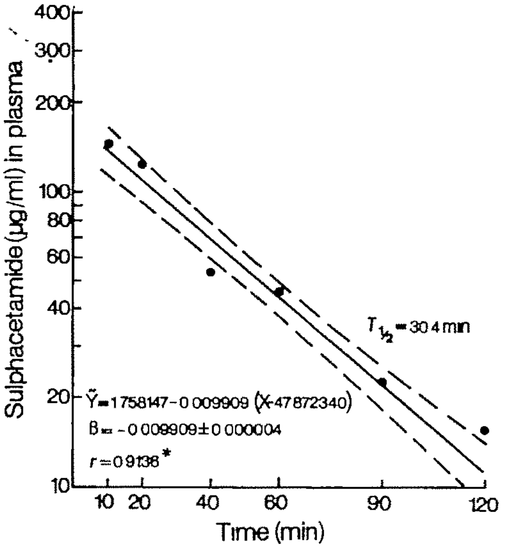


Figure 2 Relationship between the time of sulphacetamide sodium administration, 200 mg/kg and the log of sulphacetamide concentration in the plasma. The solid line is the estimated regression line: $\hat{y}=\bar{y}+\beta(x-\bar{x})$. The dotted lines are the 95% confidence limits. The correlation coefficient (r) is significant. Each point represents the mean of 5 to 6 mice.

In order to assess the effect of cancer on drug absorption, sulphacetamide remaining in the stomach and the intestine was measured (Figure 6). Within the first 20 min following drug administration, more sulphacetamide was recovered from the stomach and the intestine of leukaemic mice.

The area under the curve of sulphacetamide plasma/concentration time curve (Figure 1) of control and leukaemic mice were compared. The AUC was found to be significantly larger in leukaemic mice than in controls: $\bar{D}=12778.04$; $S_{\bar{D}}=1372.16$; $t=9.31$; $f=35$; $P\leqslant0.05$.

Discussion

Taking into consideration the higher metabolic rate and the faster circulation time in mice, the kinetics of sulphacetamide tissue distribution in this species is not unlike that observed in rats (Nadeau & Marchand, 1975). Concentrations of sulphacetamide as a function of time in the liver and muscle reflect those observed in the plasma. In the brain and fat tissue, the kinetics of sulphacetamide had a more specific pattern. However, contrary to what has been found in the rat, equilibrium between plasma sulphacetamide and other tissues was reached very slowly. Furthermore, a

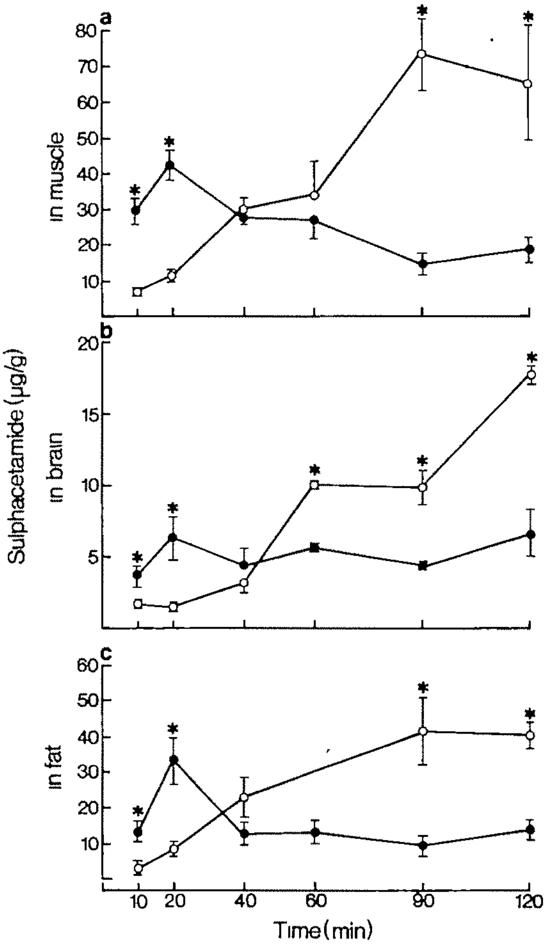


Figure 3 Sulphacetamide concentration in (a) muscle, (b) brain and (c) fat tissue at different times after oral administration of sulphacetamide sodium, 200 mg/kg, in control (●) and leukaemic mice (L-1210) (○), 5 days after inoculation. Each point represents the mean of results from 5 to 6 mice. Vertical lines show standard error. *$P < 0.05$.

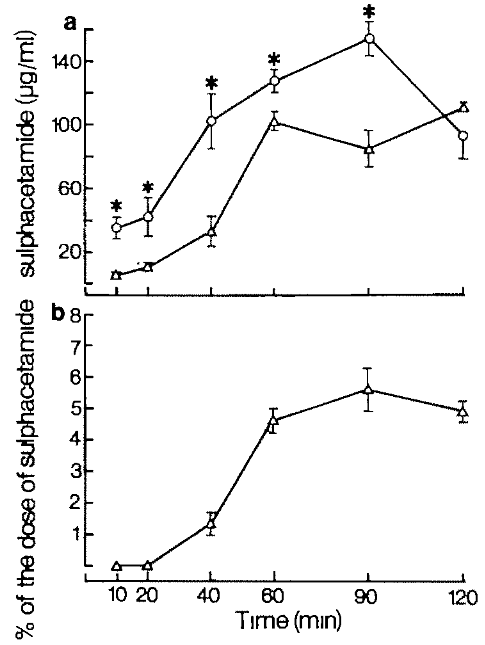


Figure 4 (a) Sulphacetamide concentration in the plasma (○) and ascitic fluid (△) and (b) accumulation in the ascitic fluid (△) at different times after oral administration of sulphacetamide sodium, 200 mg/kg in leukaemic mice (L-1210), 5 days after inoculation. Each point represents the mean results from 5 to 6 mice. Vertical lines show standard error. *$P < 0.05$.

tissue/plasma ratio of one was reached in the muscle, liver and spleen of the mice whereas, in the rat, a ratio higher than 0.7 could never be found during the same period of observation.

The marked difference in plasma sulphacetamide kinetics in control and leukaemic mice may be partially explained by delay in gastrointestinal absorption in the leukaemic mice. The elimination phase of the drug was reached within the first 10 min in controls but not until the 90 min period in leukaemic animals. The hypothesis of delayed gastrointestinal absorption is reinforced by the greater amount of sulphacetamide recovered from the stomach and the intestine of leukaemic mice during the early periods following drug administration. There are many factors that could be responsible for the delay in the intestinal absorption of sulphacetamide in leukaemic animals (Wagner, 1961; Levine, 1970). One of these is the decrease in effective mucosal blood flow to the intestine (Ther & Winne, 1971) that could be associated with the emaciated state of these animals (Diamond, Doluisio & Crouthamel, 1970). However, there is also evidence that the large blood supply to the intestine makes it unlikely that diminution of blood flow may be responsible for decrease in the rate of drug absorption by passive diffusion (Varró, Blakó, Csernay, Jung & Szarvas, 1965; Lundgren, 1967). Another factor known to delay drug absorption in the intestinal tract is the slowing in gastric emptying (Bates & Gibaldi, 1970). This factor may play an important role in the delay of intestinal absorption of sulphacetamide since the stomach of leukaemic mice contained more than twice as much sulphacetamide as control animals during the first 20 min following drug administration. Biliary salts are known to increase the intestinal absorption of drugs in the dissociated form (Feldman & Gibaldi, 1969; Nightingale, Axelson &

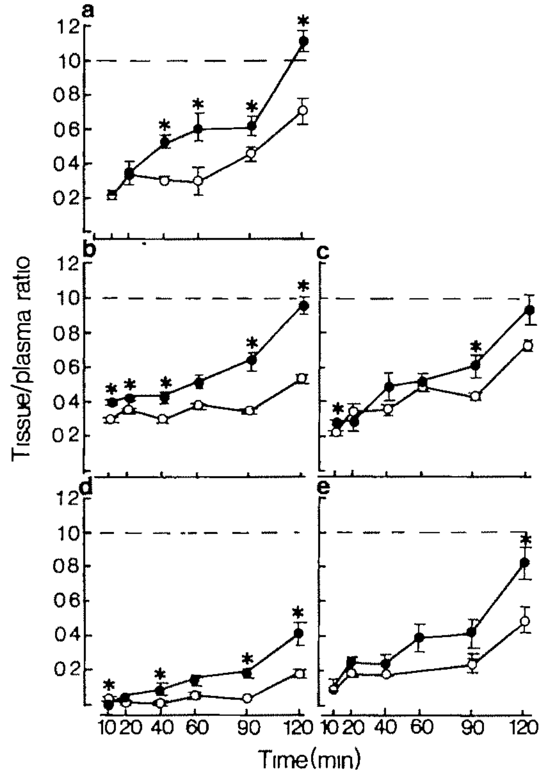


Figure 5 Tissue/plasma ratio of sulphacetamide concentration at different times after oral administration of sulphacetamide sodium, 200 mg/kg in control (●) and leukaemic mice (L-1210) (○), 5 days after inoculation: (a) muscle, (b) liver, (c) spleen, (d) brain and (e) fat tissue Each point represents the mean from results from 5 to 6 mice Vertical lines show standard error $^*P<0.05$.

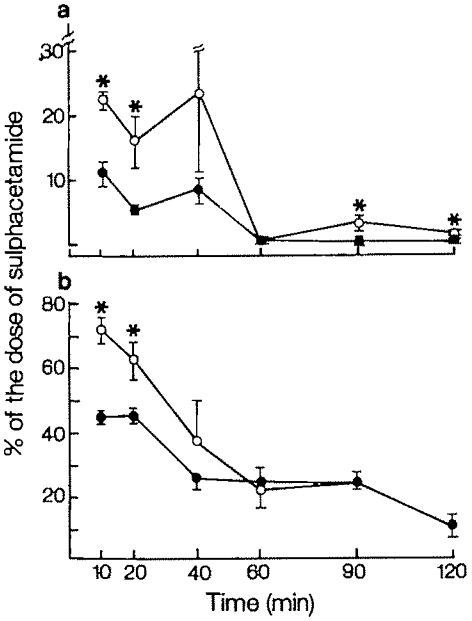


Figure 6 Percentage of the dose of sulphacetamide recovered from (a) the stomach and (b) the intestine at different times after oral administration of sulphacetamide sodium, 200 mg/kg in control (●) and leukaemic mice (L-1210) (○), 5 days after inoculation. Each point represents the mean results from 5 to 6 mice. Vertical lines show standard error. $^*P<0.05$.

Gibaldi, 1971). Although we are unaware of published data dealing with the possible decrease of biliary excretion in leukaemic animals, hepatic dysfunction has been extensively reported in tumour-bearing animals (Greenstein, 1954; Homburger, 1957). It is also well known that factors that reduce the rate of cell renewal in the small intestine of animals, like starvation and protein depletion (Hooper & Blair, 1958; Takano, 1964; Deo & Ramalingaswami, 1965), cause a shortening of villi and absorptive surface is significantly reduced (Clark & Harland, 1963; Trier & Browning, 1966). Finally, any modification in the state of the epithelial membranes could modify the rate of drug absorption in the gut; given the extensive changes in the biochemistry of cancerous animals (Greenstein, 1954), it would not be surprising that the dynamics of the membranes of columnar epithelical cell be altered and drug absorption impaired.

The role of ascitic fluid in these changes of the kinetics of sulphacetamide observed in leukaemic mice is difficult to assess. Since there is little sulphacetamide recovered from the ascitic fluid within the first 20 min, it cannot explain the marked difference in tissue sulphacetamide concentrations observed during that period. However, ascitic fluid could act as an important reservoir for plasma sulphacetamide. This could explain the high drug concentrations observed during the last 60 min of the study.

Although sulphacetamide is apparently not metabolized to a great extent in the rat (Millburn, Smith & Williams, 1967; Marchand & Nadeau, 1973), there is no evidence that the mouse is similar to the rat in that respect. Inhibition of drug metabolizing enzymes in leukaemic mice could possibly explain the high plasma concentration of the drug in the late periods of observation (Kato *et al.*, 1963; Wilson, 1968, 1971; Rosso *et al.*, 1971). Impairment of renal function cannot be ruled out as a factor responsible for the high concentration of sulphacetamide in the plasma of leukaemic mice, one to two hours after drug administration. Also, since these animals are likely to be in a catabolic state (Begg, 1958), a lowering in urine pH would also favour tubular reabsorption of sulphacetamide, pK 5.4 (Dettli & Spring, 1973). An increase in plasma protein, or affinity could possibly decrease sulphacetamide kidney filtration but opposite observations were made in rats: a decrease in plasma protein, as well as an increase in plasma free sulphacetamide were observed (Nadeau & Marchand, 1975).

The small concentrations of sulphacetamide in the brain of normal mice are consistent with previous findings in the rat (Goldsworthy, Aird & Becker, 1954; Nadeau & Marchand, 1975). However, contrary to these last observations, sulphacetamide concentration in the brain of control mice remained constant throughout the experimental periods whereas there was progressive accumulation of the drug in the brain of leukaemic mice. This could be interpreted as a change in the permeability of the blood brain barrier in leukaemic mice.

The lower tissue/plasma ratios observed in leukaemic mice are striking. It affects all tissues to varying degrees, and the differences are accentuated in the late periods of observation. It is difficult to explain these differences without invoking decrease in drug binding to tissue proteins in leukaemic mice (Gillette, 1973) or change in plasma and tissue pH favouring the ionization of sulphacetamide which would decrease diffusion of the drug from the plasma to tissues in leukaemic mice.

From the greater AUC in leukaemic mice, one would be tempted to conclude that drug bioavailability is increased in leukaemic mice. Although this conclusion cannot be ruled out, the greater AUC in leukaemic mice may simply be due to larger amounts of drug given to these mice because of their weights. This greater AUC could also be explained, in part, by the ascitic fluid that may be looked upon as a trap for sulphacetamide, preventing the drug from going to the cardiovascular compartment and preventing its excretion by the kidney; indeed, there seems to be a rapid equilibrium reached between drug in the plasma and the ascitic fluid.

From these and previous reports, one may conclude that the presence of cancer changes the distribution kinetics of sulphacetamide which could be due to delay in gastrointestinal absorption as well as trapping of the drug by ascitic fluid. These changes in the distribution kinetics coupled with decreased tissue/plasma ratios may have a bearing on the duration and intensity of drug action. It remains to be established that they are relevant to man.

This work was supported by the National Cancer Institute of Canada The authors wish to thank Miss Claudette Lamoureux and Miss Claudette Doyon for their able technical assistance. We wish to acknowledge the invaluable contribution of Dr U. Maag, Département d'Informatique, Université de Montréal, who suggested the statistical analyses for the difference between areas.

References

ADAMSON, R.H. & FOUTS, J.R. (1961). The metabolism of drugs by hepatic tumors. *Cancer Res.*, **21**, 667-672.

BATES, T.R. & GIBALDI, M. (1970). Gastrointestinal absorption of drugs. In *Biopharmaceutics*, ed. J. Swarbrick, pp. 57-97. Lea & Febiger, Philadelphia.

BEGG, R.W. (1958). Tumor-host relations. *Adv. Cancer Res.*, **5**, 1-54.

BRATTON, A.C. & MARSHALL, E.K. (1939). A new coupling component for sulfanilamide determination. *J. biol. Chem.*, **128**, 537-550.

CLARK, P.A. & HARLAND, W.A. (1963). Experimental malabsorption with jejunal atrophy induced by colchicine. *Br. J. exp. Path.*, **44**, 520-523.

DEO, M.G. & RAMALINGASWAMI, V. (1965). Reaction of the small intestine to induced protein malnutrition in rhesus monkeys. A study of cell population kinetics in the jejunum. *Gastroenterology*, **49**, 150-157.

DETTLI, L. & SPRING, P. (1973). Dosage regimens of sulphonamides based on pharmacokinetic parameters: principles and experimental results. Krueger Thiemer's theory on drug dosage: a simplified presentation. In *Biological Effects of Drugs in Relation to their Plasma Concentrations*, ed. D.S. Davies & B.N.C. Prichard, Baltimore, University Park Press.

DIAMOND, L, DOLUISIO, J.T. & CROUTHAMEL, W.G. (1970). Physiological factors affecting intestinal drug absorption. *Eur. J. Pharmac.*, **11**, 109-114.

FELDMAN, S. & GIBALDI, M. (1969). Bile salt-induced permeability changes in the isolated rat intestine. *Proc. Soc. exp. Biol.*, **132**, 1031-1033.

GILLETTE, J.R. (1973). The importance of tissue distribution in pharmacokinetics. *J. Pharmacokin. Biopharm.*, **1**, 497-520.

GOLDSTEIN, A. (1964). *Biostatistics, an Introductory Text.* pp. 129-147, New York, The MacMillan Co.

GOLDSWORTHY, P.D., AIRD, R.B. & BECKER, R.A. (1954). The blood-brain barrier. The effect of acidic dissociation constant on the permeation of certain sulfonamides into the brain. *J. cell Comp. Physiol.*, **44**, 519-526.

GREENSTEIN, J.P. (1954). *Biochemistry of Cancer.* pp. 507-588. Academic Press, Inc., New York.

HOMBURGER, F. (1957). *The biologic basis of cancer management.* pp. 148-150. Harper & Brothers, New York.

HOOPER, C S. & BLAIR, M. (1958). The effect of starvation on epithelial renewal in the rat duodenum. *Expl. Cell Res.*, **14**, 175-181.

KATO, R., FRONTINO, G. & VASSANELLI, P. (1963).

Decreased activities of liver microsomal drug-metabolizing enzymes in the rats bearing Walker carcinosarcoma. *Experientia,* **19,** 31-32.

KATO, R., TAKANAKA, A. & OSHIMA, T. (1968). Drug metabolism in tumor-bearing rats. (II). *In vivo* metabolisms and effects of drugs in tumor-bearing rats. *Jap. J. Pharmac.,* **18,** 245-254.

LEVINE, R.R. (1970). Factors affecting gastrointestinal absorption of drugs. *Am. J. Dig. Dis.,* **15,** 171-188.

LUNDGREN, O. (1967). Studies on blood flow distribution and countercurrent exchange in the small intestine. *Acta Physiol. Scand.,* **303** (Suppl.), 3-42.

MARCHAND, C. & NADEAU, D (1973). Effect of 2-Diethylaminoethyl-2, 2-Diphenylvalerate hydrochloride (SKF 525-A) on sulphacetamide distribution and excretion in rats. *Br. J. Pharmac.,* **47,** 69-76.

MILLBURN, P., SMITH, R L & WILLIAMS, R.T. (1967). Biliary excretion of foreign compounds; sulphonamide drugs in the rats. *Biochem. J.,* **105,** 1283-1287.

NADEAU, D & MARCHAND, C (1975). Change in the kinetics of sulphacetamide tissue distribution in Walker tumor-bearing rats. *Drug Met. & Dispos.,* **3,** 565–576.

NIGHTINGALE, C.H., AXELSON, J.E. & GIBALDI, M. (1971). Physiologic surface-active agents and drug absorption. VIII: effect of bile flow on sulfadiazine absorption in the rat. *J. Pharm. Sci.,* **60,** 145-147.

ROSSO, R., DONELLI, M.G., FRANCHI, G. & GARATTINI, S. (1971). Impairment of drug metabolism in tumor-bearing animals. *Eur. J. Cancer,* **7,** 565-577.

TAKANO, J (1964). Intestinal changes in protein-deficient rats. *Exp. & Mol. Pathol.,* **3,** 224-231.

THER, L & WINNE, D (1971). Drug absorption. *A. Rev. Pharmacol.,* **11,** 57-70.

TRIER, J.S & BROWNING, T.H. (1966). Morphologic response of the mucosa of human small intestine to X-ray exposure. *J. clin. Invest.,* **45,** 194-205.

VARRÓ, V., BLAKÓ, G., CSERNAY, L., JUNG, I. & SZARVAS, F. (1965). Effect of decreased local circulation of the absorptive capacity of a small-intestine loop in the dog. *Am. J. Dig. Dis.,* **10,** 170-177.

WAGNER, J G. (1961). Biopharmaceutics: absorption aspects. *J. Pharm. Sci.,* **50,** 359-387.

WAY, E.L., SMITH, P.K., HOWIE, D L., WEISS, R. & SWANSON, R. (1948). The absorption, distribution, excretion and fate of para-aminosalicylic acid. *J. Pharmac. exp. Ther.,* **93,** 368-382.

WELCH, B.L. (1947). The generalization of 'Student's' problem when several different population variances are involved. *Biometrika,* **34,** 28-35.

WILSON, J.T. (1968). An investigation of the decrease in the metabolism of hexobarbital, aminopyrine and *p*-nitrobenzoic acid by liver from rats bearing a pituitary mammotropic tumor. *J. Pharmac. exp. Ther.,* **160,** 179-188.

WILSON, J.T. (1971). Altered rat hepatic drug metabolism after implantation of a pituitary mammotropic tumor (M_tT), Walker carcinosarcoma or adenocarcinoma and after removal of the M_tT. *Endocrinology,* **88,** 185-194.

(Received August 5, 1975.
Revised November 7, 1975.)

Br. J. Pharmac. (1976), **56**, 431–436

EFFECTS OF MAZINDOL, A NON-PHENYLETHYLAMINE ANOREXIGENIC AGENT, ON BIOGENIC AMINE LEVELS AND TURNOVER RATE

M.O. CARRUBA, A. GROPPETTI, P. MANTEGAZZA,
L. VICENTINI & F. ZAMBOTTI
Second Chair, Department of Pharmacology, School of Medicine,
University of Milan, Via Vanvitelli 32, 20129, MILANO, Italy

1 Mazindol is a new anorexigenic agent which possesses a different chemical structure from that of phenylethylamines, but shows a pharmacological profile similar to that of (+)-amphetamine.

2 Mazindol neither altered whole brain monoamine levels (noradrenaline (NA), dopamine, 5-hydroxytryptamine (5-HT)) nor changed NA levels in the hypothalamus or dopamine levels in the caudate nucleus.

3 Mazindol enhanced dopamine turnover rate in the caudate nucleus, as shown by the increased rate of dopamine decline after blockade of catecholamine synthesis by α-methyl-*p*-tyrosine and decreased the conversion index of [^{3}H]-tyrosine into brain NA.

4 Mazindol administration did not modify pargyline-induced decline of 5-hydroxyindoleacetic acid suggesting that 5-HT turnover is not altered by this drug.

Introduction

Food intake can be suppressed by drugs, such as fenfluramine and (+)-amphetamine which have many diverse behavioural and biochemical properties but are both anorectics. Considerable evidence suggests that the anorectic effect of (+)-amphetamine is mediated by interactions with brain catecholamines (Weissman, Koe & Tenen, 1966; Holtzmann & Jewett, 1971; Schulz & Frey, 1972; Kruk, 1973; Groppetti, Barzaghi & Mantegazza, 1973a; Groppetti, Zambotti, Biazzi & Mantegazza, 1973b; Leibowitz, 1975), while fenfluramine, although chemically related to (+)-amphetamine, induces anorexia via direct or indirect indoleamine-like effects (Funderburk, Hazelwood, Ruckart & Ward, 1971; Samanin, Ghezzi, Valzelli & Garattini, 1972; Clineschmidt, 1973; Jespersen & Scheel-Krüger, 1973).

Mazindol (5-hydroxy-5-*p*-chlorophenyl-2,3-dihydro-5H-imidazo-2,1-a-isoindole) (Figure 1), a drug with a different chemical structure from those of the β-phenylethylamines, was found in our laboratories to possess a behavioural profile similar to that of (+)-amphetamine (Zambotti, Carruba, Barzaghi, Vicentini, Groppetti & Mantegazza, 1975). In fact mazindol, when given to rats, in addition to causing anorexia, increased motor activity and body temperature, elicited stereotyped behaviour and caused turning in animals with unilateral nigro-striatal lesions. Thus, it seemed of interest to investigate whether mazindol and (+)-amphetamine share not only their common behavioural profile, but also similar biochemical mechanisms. To this purpose, experiments on biogenic amine levels in whole brain and in discrete brain areas have been performed. In addition, because of the results obtained from these preliminary experiments, we decided to study the action of behaviourally active doses of mazindol on the turnover rate of brain 5-hydroxytryptamine (5-HT), noradrenaline (NA) and striatal dopamine.

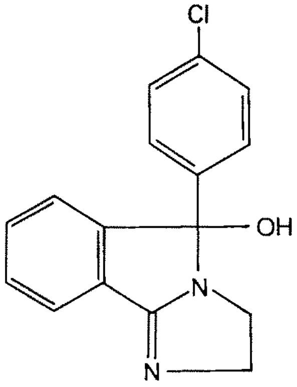


Figure 1 Chemical structure of mazindol.

Methods

All the experiments were performed on male Sprague-Dawley rats (Charles River) (150–200 g). Animals were killed by decapitation, the brain was removed, and in some instances the hypothalamus or caudate nucleus was dissected out, frozen on dry ice and stored at −20°C until assayed. Details of dose and time are given in the relevant places throughout the paper.

Determination of catecholamines

The tissues were homogenized in ice-cold 0.4 N perchloric acid containing 0.1% metabisulphite ($Na_2S_2O_5$) and centrifuged. The catecholamines in the supernatant fluid were absorbed onto Al_2O_3 and then extracted with 0.2 N acetic acid as described by Brodie, Comer, Costa & Dlabac (1966a) and assayed fluorimetrically according to the method of Chang (1964).

Determination of 5-hydroxyindoleacetic acid and 5-hydroxytryptamine

The brain was homogenized with 10 volumes of acidified butanol and centrifuged. The butanol phase was used for 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) determination as described by Curzon & Green (1970).

Measurement of dopamine turnover

Rats were injected intraperitoneally with 300 mg/kg of α-methyl-*p*-tyrosine methylester hydrochloride (AMPT) 15 min after mazindol (5 or 10 mg/kg, s.c.) or 0.9% w/v NaCl solution (saline) and killed 1.5 and 3 h thereafter. The caudate nucleus was then immediately dissected from the brain and stored frozen at −20°C until assayed. The frozen tissue samples were homogenized in 0.4 N perchloric acid and dopamine was assayed as previously described. The turnover rate of caudate dopamine was calculated from the product of the steady state of dopamine and the rate constant of dopamine decline (Brodie, Costa, Dlabac, Neff & Smookler, 1966b).

Measurement of noradrenaline turnover

[3,5-^{3}H]-L-tyrosine was diluted in saline so that an injection volume of 0.5 ml per 100 g body weight corresponded to 1.0 mCi/kg. Mazindol was injected subcutaneously 40 min before the intravenous injection of [^{3}H]-tyrosine. Animals were killed 20 min after the injection of the labelled compound. Brain samples were homogenized in 0.4 N perchloric acid containing 0.1% sodium metabisulphite. The homogenate was centrifuged and 4.7 ml of the supernatant fluid were removed and adjusted to pH 4.2 by addition of 0.3 ml of 10M potassium acetate. After a second centrifugation, 4 ml of the clear supernatant fluid was placed on a Dowex AG 50Wx4, 200–400 mesh, column (25 mm x 25 mm^2) prepared as described by Haggendal (1963). Elution of the columns and radiometric and fluorimetric assay of NA and tyrosine were done as described by Neff, Spano, Groppetti, Wang & Costa (1971). The conversion index (CI) of the [^{3}H]-tyrosine into brain NA was determined by dividing the radioactivity of the amine (d min^{-1} g^{-1}) by the specific activity (sp. act.) of tyrosine (Ty) (Costa *et al.*, 1971).

$$CI = \frac{\text{d min}^{-1}\,\text{g}^{-1}\,(\text{NA})}{\text{sp. act. (Ty)}}$$

Measurement of 5-hydroxytryptamine turnover

Brain 5-HT turnover rate was calculated, as described by Neff, Tozer & Brodie (1966), from the decline of brain 5-HIAA after intraperitoneal administration of

Table 1 Whole brain monoamine concentrations (noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT)) in the rat after administration of different doses of mazindol

Treatment	*Dose (mg/kg s.c.)*	*Time* (h)*	*NA*	*DA (μg/g ± s.e.)*	*5-HT*
Saline	—	—	0.43 ± 0.007	1.08 ± 0.024	0.58 ± 0.017
Mazindol	2.5	1	0.47 ± 0.018	1.07 ± 0.040	0.59 ± 0.021
	5.0	1	0.45 ± 0.020	1.07 ± 0.012	0.55 ± 0.021
	10.0	1	0.42 ± 0.010	1.08 ± 0.022	0.59 ± 0.011
•	5.0	3	0.40 ± 0.014	1.00 ± 0.020	0.56 ± 0.016
	2.5	5	0.45 ± 0.016	1.18 ± 0.043	0.62 ± 0.032
	5.0	5	0.47 ± 0.010	1.15 ± 0.053	0.56 ± 0.032
	10.0	5	0.41 ± 0.005	1.11 ± 0.052	0.57 ± 0.018

* Time after the drug administration when animals killed. Each value represents the mean ± s.e. of 10–20 rats.

90 mg/kg pargyline alone or 10 min after mazindol 5 mg/kg subcutaneously. Animals were killed 30 and 60 min after pargyline administration.

Statistics

Data were analyzed using Student's *t* test; the level of significance was chosen as $P=0.05$. The method of least squares was used to calculate the best-fit curves. Furthermore an analysis of variance has been performed in order to evaluate the significance of the regression and parallelism.

Drugs

Drugs were dissolved in 0.9% w/v NaCl solution (saline) unless otherwise stated. The injection volume was always 0.5 ml per 100 g body weight. For mazindol the doses refer to the base, for the other drugs to the salt. α-Methyl-L-*p*-tyrosine methylester hydrochloride and pargyline hydrochloride were generously supplied by Hoffman-La Roche, Research Division, Basel, and mazindol by Sandoz, Milan. [3,5-^{3}H]-L-Tyrosine (53 Ci/mmol) was obtained from the Radiochemical Centre, Amersham.

Results

Tissue concentrations of monoamines

The concentrations of NA, dopamine and 5-HT in brain tissue at different times after the subcutaneous injection of various doses of mazindol (2.5, 5 or 10 mg/kg, s.c.) are shown in Table 1. These data indicate that brain amine levels were not significantly different from those of the controls at any dose and any time studied. Similarly, administration of the same doses of mazindol did not alter the hypothalamic NA content when measured 1 h after drug treatment (Table 2). Table 3 shows that the concentrations of caudate dopamine were also unaffected after injection of mazindol, 2.5, 5 or 10 mg/kg subcutaneously over a 5 h period.

*Effect of mazindol on rate of caudate dopamine decline after α-methyl-*p*-tyrosine*

Table 4 shows the effect of mazindol on the decline of caudate dopamine concentrations elicited by AMPT. In saline-treated rats, after blockade of catecholamine synthesis with AMPT (300 mg/kg, i.p.), the caudate dopamine concentrations declined exponentially with a rate constant of 0.28 ± 0.027 (h^{-1}). Fifteen min after mazindol administration (5 or 10 mg/kg, s.c.) caudate dopamine levels were unaffected and the rate of the dopamine decline after AMPT was statistically higher than that of controls only when 10 mg/kg was injected (Table 4).

Effect of mazindol on turnover rate of brain noradrenaline

The effects of mazindol and saline on the CI of [^{3}H]-tyrosine into brain NA were compared. The results shown in Table 5 indicate that mazindol at a dose of 2.5 and 5 mg/kg subcutaneously did not change the concentrations and the sp. act. of brain tyrosine and did not alter steady state levels of brain NA of rats receiving the labelled compound 40 min after the drug. Mazindol at these doses decreased the CI of tyrosine into NA and the effect was statistically significant and dose-related.

Table 2 Hypothalamic noradrenaline (NA) concentrations in the rat after acute administration of different doses of mazindol

Treatment	*Dose (mg/kg s.c.)*	*NA * (μg/g ± s e.)*
Saline	—	2.57 ± 0.06
Mazindol	2.5	2.37 ± 0.15
	5.0	2.68 ± 0.10
	10.0	2.46 ± 0.16

* One hour after treatment. Each value represents the mean ± s.e. of 5 animals.

Table 3 Effect of mazindol on caudate dopamine concentrations in the rat.

Dose of mazindol (mg/kg s.c.)	*Dopamine (hours after treatment)* 1	3	5
2.5	110 ± 4.5	—	89 ± 2.8
5.0	103 ± 6.6	108 ± 2.9	99 ± 6.3
10.0	107 ± 2.1	—	100 ± 4.9

The figures indicate mean values ± s.e. for 8 rats and are expressed as % of control values. Control values: dopamine 10.28 ± 0.44 μg/g.

Effect of mazindol on 5-hydroxyindoleacetic acid rate of decline after pargyline

The rate of decline of brain 5-HIAA after treatment with pargyline (90 mg/kg, i.p.), as is shown in Table 6, could not be altered by administration of mazindol, (5 mg/kg, s.c.). Calculation of turnover rate constants gave a value of $K=0.75\pm0.03$ (h^{-1}) for saline-treated controls that was not statistically different from that obtained in mazindol-pretreated animals ($K=0.84\pm0.02$ h^{-1}).

Discussion

The brain biogenic amines (NA, dopamine, 5-HT) levels were measured in rats receiving behaviourally active doses of mazindol over a 5 h time period. None of the doses injected caused a change of these amine concentrations at any time studied. No changes were detected even when NA and dopamine levels were measured in the hypothalamus and in the caudate nucleus respectively. Thus, these results indicate that mazindol is not similar to (+)-

Table 4 Turnover rates and turnover times of caudate dopamine, measured from dopamine decline, after blockade of synthesis by α-methyl-*p*-tyrosine (AMPT) (300 mg/kg i.p.)

Treatment	*Steady-state levels ($\mu g/g\pm s.e.$)*	*Rate constant of amine loss ($K(h^{-1})\pm s.e.$)*	*Turnover time†* *(h)*	*Turnover rate†* *($\mu g\ g^{-1}\ h^{-1}$)*
Controls	9.25±0.30	0.28±0.027	3.5	2.64
Mazindol‡ (5 mg/kg s.c.)	10.02±0.26	0.37±0.030	2.6	3.73
Controls	11.63±0.43	0.29±0.032	3.4	3.39
Mazindol‡ (10 mg/kg s.c.)	12.02±0.34	0.55±0.035*	1.8	6.61

* $P<0.01$.

† Turnover rate is the product of the steady state level and *K*, the rate constant of dopamine decline; turnover time is 1/*K*. ‡ Mazindol was given 15 min before AMPT treatment.

Table 5 Effect of mazindol on conversion of [3,5-^{3}H]-L-tyrosine into brain noradrenaline (NA)

Treatment	*Dose (mg/kg s.c.)*	*No. of rats*	*Tyrosine (d min^{-1} nmol^{-1} ±s.e. × 10^{-3})*	*NA (nmol/g ± s.e.)*	*Conversion index (NA nmol g^{-1} 20 min^{-1} ± s.e.)*
Saline	—	7	5.24±0.28	2.40±0.13	0.56±0.06
Mazindol	2.5	8	5.97±0.23	2.49±0.11	0.38±0.02*
Saline	—	7	3.69±0.08	1.95±0.08	0.49±0.03
Mazindol	5.0	6	4.22±0.31	2.04±0.08	0.22±0.01†

* $P<0.02$; † $P<0.001$

Table 6 Turnover rate of brain 5-hydroxytryptamine (5-HT) and turnover time of 5-hydroxyindoleacetic acid (5-HIAA) as measured from the decline of 5-hydroxyindoleacetic acid after pargyline

Treatment	*Steady-state brain level of 5-HIAA* *($\mu g/g\pm s.e.$)*	*Rate constant of 5-HIAA loss after pargyline* *($K(h^{-1})\pm s.e.$)*	*Turnover time of 5-HIAA* *(h)*	*Turnover rate of 5-HT* *($\mu g\ g^{-1}\ h^{-1}$)*
Controls	0.662±0.04	0.754±0.036	1.32	0.4994
Mazindol (5 mg/kg s.c.)	0.717±0.03	0.845±0.025	1.18	0.6063

amphetamine or fenfluramine since (+)-amphetamine decreases tissue concentrations of NA (McLean & McCartney, 1961; Moore & Lariviere, 1963; Carr & Moore, 1969; Groppetti & Costa, 1969; Costa, Groppetti & Revuelta, 1971) while fenfluramine reduces both 5-HT and NA concentrations of brain tissue (Duce & Gessa, 1966; Opitz, 1967; Duhault & Verdavainne, 1967; Costa *et al.*, 1971).

Since changes in the turnover rates of neurohormones are believed to be a better index of neuronal activity than changes in the concentrations of the amines, which might remain virtually unchanged despite marked changes in their rates of synthesis, turnover studies of brain NA, 5-HT and striatal dopamine were performed. The data obtained clearly show that mazindol, like (+)-amphetamine (Groppetti, Misher, Naimzada, Revuelta & Costa, 1972; Groppetti *et al.*, 1973b) increases the dopamine turnover rate in the caudate nucleus. However, in contrast to (+)-amphetamine and fenfluramine (Groppetti *et al.*, 1972; Groppetti *et al.*, 1973b) mazindol decreases the incorporation rate of [^{3}H]-tyrosine into brain NA in a dose-related manner. Furthermore, in our experimental conditions, mazindol did not modify the rate constant of 5-HIAA decline following pargyline administration. In a previous report (Zambotti *et al.*, 1975), on the basis of the observation that the anorectic effect of mazindol can be antagonized by pretreatment with AMPT, an inhibitor of catecholamine synthesis (Spector, Sjoerdsma & Udenfriend, 1965), and with pimozide, a selective dopamine blocker (Andén, Butcher, Corrodi, 1970), we pointed out that a presynaptic dopaminergic mechanism resulting in increased dopamine release might be involved in mazindol-induced anorexia. Further support for the suggestion that mazindol causes dopamine release comes from the finding that rats with unilateral lesions in the substantia nigra began to rotate to the side of the lesion (behaviour related to dopamine release in the striatum of the non-lesioned side) after the administration of mazindol (Zambotti *et al.*, 1975). The present data on dopamine turnover also confirm this hypothesis on a biochemical basis and are consistent with an observation of Jori & Dolfini (1974) who found an increase in the brain homovanillic acid concentration after 15 mg/kg of mazindol. Thus, we might conclude that the mechanisms whereby mazindol induces anorexia and turning behaviour depend upon an increased synthesis and release of dopamine from its nerve terminals. Among the several possibilities for the effects of mazindol on NA, the one that appears most likely is that the deceleration of NA turnover by mazindol could be the result of an increased receptor stimulation which, through a negative feed-back mechanism, inhibits NA biosynthesis in the presynaptic neurones. It has been recently reported, in fact, by Engstrom, Kelly & Gogerty (1975) that mazindol is able to block the neuronal uptake of NA, as shown by the reduction in uptake of intraventricularly injected [^{3}H]-NA.

The failure to detect any alteration of the pargyline-induced decline of brain 5-HIAA after mazindol treatment can be taken as evidence that this drug does not modify 5-HT turnover. Based on this finding, it seems possible to rule out for mazindol-induced anorexia, the same mechanism of action suggested for fenfluramine (Funderburk *et al.*, 1971; Samanin *et al.*, 1972; Jespersen & Scheel-Krüger, 1973; Clineschmidt, 1973). In conclusion, mazindol, although having a behavioural profile similar to that of (+)-amphetamine (Zambotti *et al.*, 1975) shows some differences in its neurochemical effects. Moreover the data reported here show that mazindol does not share any biochemical effects with fenfluramine.

This work was supported by C.N.R. grant No. 74.00222. 04.115.2380.

References

ANDÉN, N.E., BUTCHER, S.G., CORRODI, M., FUXE, K. & UNGERSTEDT, U. (1970). Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *Eur. J. Pharmac.*, **11**, 303–314.

BRODIE, B.B., COMER, M.S., COSTA, E. & DLABAC, A. (1966a). The role of brain serotonin in the mechanism of the central action of reserpine. *J. Pharmac. exp. Ther.*, **152**, 340–349.

BRODIE, B.B., COSTA, E., DLABAC, A., NEFF, N.H. & SMOOKLER, H.H. (1966b). Application of steady state kinetics to the estimation of synthesis rate and turnover time of tissue catecholamines. *J. Pharmac. exp. Ther.*, **154**, 493–498.

CARR, L.A. & MOORE, K.E. (1969). Norepinephrine: release from brain by d-amphetamine *in vivo*. *Science*, **164**, 322–323.

CHANG, C.C (1964). A sensitive method for spectrophotofluorimetric assay of catecholamines. *Int. J. Neuropharmac.*, **3**, 643–649.

CLINESCHMIDT, B.V. (1973). 5,6-Dihydroxytryptamine: suppression of the anorexigenic action of fenfluramine. *Eur. J. Pharmac.*, **24**, 405–409.

COSTA, E., GROPPETTI, A., REVUELTA, A. (1971). Action of fenfluramine on monoamine stores of rat tissues. *Br. J. Pharmac.*, **41**, 57–64.

CURZON, G. & GREEN, A.R. (1970). Rapid method for the determination of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in small regions of the rat brain. *Br. J. Pharmac.*, **39**, 653–655.

DUCE, M. & GESSA, G.L (1966). Deplezione di catecolamine centrali e periferiche indotta da Fenfluramina. *Boll. Soc. It. Biol. Sper.*, **42**, 1631–1637.

DUHAULT, J. & VERDAVAINNE, C. (1967). Modification du taux de serotonine cérebrale chez le rat par les trifluoromèthyl-phènyl-2-ethylamino propane (Fenfluramine 7685). *Arch. Int. Pharmacodyn. Thér.*, **170**, 276–286.

ENGSTROM, R.G., KELLY, L.A. & GOGERTY, J.H (1975). The effects of 5-hydroxy-5-(4-chlorophenyl)-2,3-Dihydro-5H-imidazo (2,I-a) isoindole (Mazindol, 5aH 42-548) on the metabolism of brain norepinephrine. *Arch. Int. Pharmacodyn.*, **214**, 308–321.

FUNDERBURK, W.H., HAZELWOOD, J.C., RUCKART, R.T. & WARD, J.W. (1971). Is 5-hydroxytryptamine involved in the mechanism of action of fenfluramine? *J. Pharm. Pharmac.*, **23**, 468–469.

GROPPETTI, A. & COSTA, E. (1969). Tissue concentrations of p-hydroxynorephedrine (pOHNE) injected with d-amphetamine: Effect of pretreatment with desipramine (DMI). *Life Sci.*, **8**, 653–665.

GROPPETTI, A., MISHER, A., NAIMZADA, M., REVUELTA, A. & COSTA, E (1972). Evidence that in rats I-Benzyl-β-Metoxy-3-trifluoromethylphenethylamine (SK&F I-39728) dissociates anorexia from central stimulation and action on brain monoamine stores. *J. Pharmac. exp. Ther.*, **182**, 464–473.

GROPPETTI, A, BARZAGHI, F. & MANTEGAZZA, P. (1973a). Ruolo delle catecolamine nell'anoressia da amfetamina. *Riv. Farmacol. Terap.*, **4**, 111a–120a.

GROPPETTI, A., ZAMBOTTI, F., BIAZZI, A. & MANTEGAZZA, P. (1973b). Amphetamine and cocaine on amine turnover. In *Frontiers in Catecholamine Research*, ed. Usdin, E. & Snyder, S.H., pp. 917–925. Oxford: Pergamon Press.

HAGGENDAL, J. (1963). An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues. *Acta physiol. scand.*, **59**, 242–254.

HOLTZMAN, S.G. & JEWETT, R.E. (1971). The role of brain norepinephrine in the anorexic effect of dextro-amphetamine and monoamine oxidase inhibitors in the rat. *Psychopharmacologia*, **22**, 151–161.

JESPERSEN, S. & SCHEEL-KRÜGER, J. (1973). Evidence for a difference in mechanism of action between fenfluramine and amphetamine-induced anorexia. *J. Pharm. Pharmac.*, **25**, 49–54.

JORI, A. & DOLFINI, E. (1974). On the effect of anorectic drugs on striatum Homovanillic acid in rats. *Pharmac. Res. Comm.*, **6**, 175–178.

KRUK, Z.L. (1973). Dopamine and 5-hydroxytryptamine inhibit feeding in rats. *Nature New Biol.*, **246**, 52–53.

LEIBOWITZ, S.F. (1975). Amphetamine: possible site and mode of action for producing anorexia in the rat. *Brain Res.*, **84**, 160–167.

McLEAN, J.R. & McCARTNEY, M. (1961). Effect of d-amphetamine on rat brain noradrenaline and serotonin. *Proc. Soc. exp. Biol. Med.*, **107**, 77–79.

MOORE, K.E. & LARIVIERE, E.W. (1963). Effects of d-amphetamine and restraint on the content of norepinephrine and dopamine in rat brain. *Bioch. Pharmac.*, **12**, 1283–1288.

NEFF, N.H., TOZER, T.N., BRODIE, B.B (1966). Application of steady state kinetics to the synthesis rate and turnover time of serotonin in the brain of normal and reserpine-treated rats. *J. Pharmac. exp. Ther.*, **153**, 177–182.

NEFF, N H., SPANO, P.F., GROPPETTI, A., WANG, C.T. & COSTA, E. (1971). A simple procedure for calculating the synthesis rate of norepinephrine, dopamine and serotonin in rat brain. *J. Pharmac. exp. Ther.*, **176**, 701–710.

OPITZ, K. (1967). Anorexigene phenylalkylamine und Serotoninstoffwechsel. *Naunyn-Schmiedebergs Arch. Pharmak. exp. Path.*, **259**, 56–65.

SAMANIN, R., GHEZZI, D., VALZELLI, L. & GARATTINI, S. (1972). The effects of selective lesioning of brain serotonin or catecholamine containing neurones on the anorectic activity of fenfluramine and amphetamine. *Eur. J. Pharmac.*, **19**, 318–322.

SCHULZ, R. & FREY, H.H. (1972). Study into the mechanism of the anorectic action of amphetamine and its p-chloro analogue. *Acta pharmac. et toxicol.*, **31**, suppl. 1, 12.

SPECTOR, S., SJOERDSMA, A. & UDENFRIEND, S (1965). Blockade of endogenous norepinephrine synthesis by α-methyl-tyrosine, an inhibitor of tyrosine hydroxylase. *J. Pharmac. exp. Ther.*, **147**, 86–95.

WEISSMAN, A., KOE, B.K. & TENEN, S.S. (1966). Antiamphetamine effects following inhibition of tyrosine hydroxylase. *J. Pharmac. exp. Ther.*, **151**, 339–352.

ZAMBOTTI, F, CARRUBA, M.O., BARZAGHI, F., VICENTINI, L., GROPPETTI, A & MANTEGAZZA, P. (1975). Behavioural effects of a new nonphenylethylamine anorexigenic agent: Mazindol. *Eur. J. Pharmac.*, (in press).

*(Received August 6, 1975.
Revised November 6, 1975.)*

Br. J. Pharmac. (1976), **56**, 437–441

THE UPTAKE AND SUBCELLULAR DISTRIBUTION OF RADIO-LABELLED METABOLITES OF DIGITOXIN IN THE GUINEA-PIG ISOLATED PERFUSED HEART

S. DUTTA,[1] B.H. MARKS[1] & P.M. STEPHEN[2]
Department of Pharmacology, Ohio State University College of Medicine,
Colombus, Ohio, 43210, U.S.A.

1 Comparisons were made of the uptake and inotropic effects of concentrations of 0.1 µM of digitoxin and its cleavage products digitoxigenin-bis-digitoxoside, digitoxigenin-mono-digitoxoside and digitoxigenin in the isolated perfused hearts of guinea-pigs.

2 Digitoxin produced the greatest inotropic responses in this series, while the sequence of cleavage products produced progressively smaller responses.

3 The uptake of digitoxin was significantly higher than that of the three metabolites, and the uptake of metabolites became progressively less with cleavage. The highest binding in each case was found in the microsomal fraction.

4 The uptake of all four digitaloids was reduced when the potassium in the perfusion medium was increased.

Introduction

The importance of the sugar component for the pharmacodynamic properties of the cardiac glycosides has been emphasized by Repke (1963). The sugars linked directly to digitoxigenin are desoxy sugars. Digitoxin undergoes stepwise cleavage yielding the corresponding genin, although *in vivo* the genin cannot usually be detected, possibly because the biotransformation as well as excretion of the released genin is far more rapid than the cleavage of the glycosides (Repke, 1959a,b; Lauterbach & Repke, 1960). In *in vivo* studies, Repke (1963) demonstrated that when digitoxin was injected in rats, in 10 min almost 5% of the material recovered from the liver was digitoxigenin-bis-digitoxoside, after a loss of one sugar molecule. The sugars alone are without any cardiotonic action (Straub, 1916) while the genins appear to be biologically active with a shorter latent period than their glycosides (Walther, 1943; Modell, Kwitt, Dayrit, Shane & Gold, 1948).

Considering the possibility of digitoxin cleavage, therefore, a systematic study was undertaken to compare the properties of digitoxin with those of digitoxigenin bis-digitoxoside, digitoxigenin mono-digitoxoside and digitoxigenin by evaluating their uptakes by perfused guinea-pig heart, their distribution in the subcellular ventricular fractions and their cardiotonic effects. Normal and high-potassium media were used.

Methods

Studies on the isolated perfused hearts of guinea-pigs were carried out with various digitoxosides of digitoxigenin, obtained from Boehringer. These were randomly tritiated by New England Nuclear Corp. using the catalytic exchange process. [^{3}H]-digitoxin was purchased from New England Nuclear Corp. All these tritiated derivatives were subjected to liquid/liquid chromatography (Repke, 1959a) which revealed one primary radioactive spot for each compound giving a R_F value characteristic for the unlabelled standards. The uptake of these glycosides by the heart tissue and their distribution in the various subcellular components was studied using the perfusion technique described by Dutta, Goswami, Lindower & Marks (1968). Langendorff perfusion of guinea-pig hearts was carried out using a peristaltic pump to deliver 4.5 ml/min of Krebs-Henseleit buffer (K-H) at 28°C (pH 7.4) through the cannulated aorta. The atria were removed to establish an idioventricular rhythm of 60-80 beats per min after which the heart was paced by bi-polar electrodes on the ventricle using threshold voltage to drive at 120 beats/minute. After an adequate period of equilibration, the preparation was observed for a control period of 30 min following which the medium was changed to an identical one containing a concentration of 0.1 µM of the tritiated cardiac glycoside under study.

---

[1] Present address: Dept of Pharmacology, Wayne State University School of Medicine, Detroit, Michigan 48201, U.S.A.

[2] Present address: Dept of Pharmacology, Christian Medical College, Vellore-632002, India.

In order to make an evaluation of the electrical and mechanical performances of the perfused heart on a four-channel polygraph, continuous records were maintained of these parameters: (1) isometric developed tension (d*T*) from a constant resting tension of 5.0 g recorded from the apex of the heart using a Grass force displacement transducer FTO3C; (2) the first derivative of the d*T* (d*f*/d*t*); (3) the perfusion pressure using a Statham transducer; (4) the surface electrocardiogram. (1) was used for the comparison of drug effects, (2) paralleled (1) and is not reported separately, and (3) and (4) provided control information.

Drug perfusion was continued for 64 min at the end of which normal K-H was perfused for 8 min to wash out the drug from the vascular and interstitial spaces so that the cardiac drug content measured would primarily represent the cellular accumulation of the drug. The effect of increased levels of potassium on the uptake of these glycosides was determined by perfusing other groups of hearts for 64 min with a concentration of 0.1 µM of the tritiated drug in K-H modified to contain 1.5 times the normal concentration of potassium (8.7 mM). These perfusions also were preceded by a control period of 30–40 min and followed by an 8 min wash-out period in normal K-H.

At the end of the wash-out, the ventricular tissue was homogenized in sucrose-disodium edetate (EDTA) to obtain a 10% suspension. The various subcellular fractions—nuclear, mitochondrial, microsomal and supernatant fractions were obtained by differential centrifugation as described by Dutta & Marks (1969). The tritiated drug content in the several fractions and in the total heart homogenate was counted in a liquid scintillation counter. The protein content of aliquots of each fraction and of the homogenate was estimated by a biuret method (Gornall, Bardawill & David, 1949), so that the concentration of the drug in the various fractions could be expressed as picomoles/mg protein. Four hearts were used for each drug and control perfusion experiment. The criterion for significance in all experiments was taken as a probability of chance occurrence of less than 5% ($P < 0.05$).

Results

Experiments with digitoxigenin and its digitoxosides in normal K-H buffer

Figure 1 presents the sequence of events during the time course of the perfusion with digitoxigenin and its various digitoxosides. Digitoxin produced a mean positive inotropic response of 48.7 ± 4.6% over the control, reached in 18–20 min and maintained until the end of the drug-perfusion period. The digitoxigenin bis-digitoxoside produced a 41 ± 3.7% response in 28 min, the mono-digitoxoside a 36 ± 4.3% response in 31 min and digitoxigenin produced the least effect of 14 ± 1.6% in 26 min; with all the drugs, plateaus of increased developed tension were maintained throughout the remainder of the drug perfusion period.

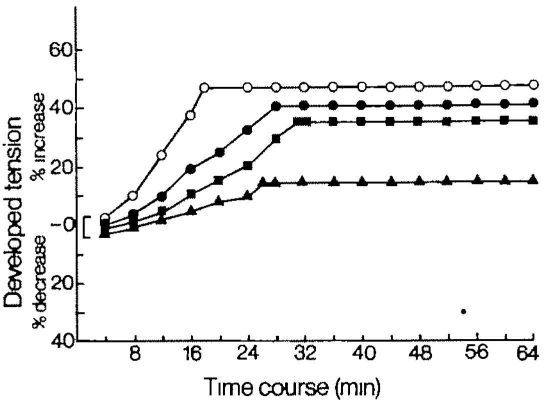


Figure 1 The developed tension (d*T*) during the 64 min perfusion of isolated hearts of guinea-pigs with digitoxin or its metabolites (all 0.1 µM) in normal Krebs-Henseleit buffer at a flow rate of 4.5 ml/min and temperature of 28°C. (○) Digitoxin; (●) digitoxigenin bis-digitoxoside; (■) digitoxigenin mono-digitoxoside, (▲) digitoxigenin. Each plot denotes mean value from 4 hearts.

The total uptake by the heart of digitoxin was 70% greater than that of the three metabolites, there being no significant difference among the quantitative uptakes of the metabolites (Table 1). The overall distribution of the drugs between the particulate and supernate fractions calculated as the supernate/pellet ratio showed a range between 0.5 to 0.77 for the four drugs, with digitoxin having the highest ratio and mono-digitoxoside the lowest ratio. Subcellular distribution showed the highest concentrations of binding in the microsomal fraction. The digitoxin accumulation in the microsomal fraction was again significantly higher than that of the metabolites, with no distinct difference among the three metabolites.

Experiments with digitoxosides in high potassium K-H buffer

Control experiments were done without any drug, but with increased potassium (1.5 × normal = 8.7 mM) in the perfusing medium for the full perfusion period of 64 minutes. On changing the perfusion medium from normal K-H to that of K-H with increased potassium at 0 time, there was a fall of 46% in the isometric

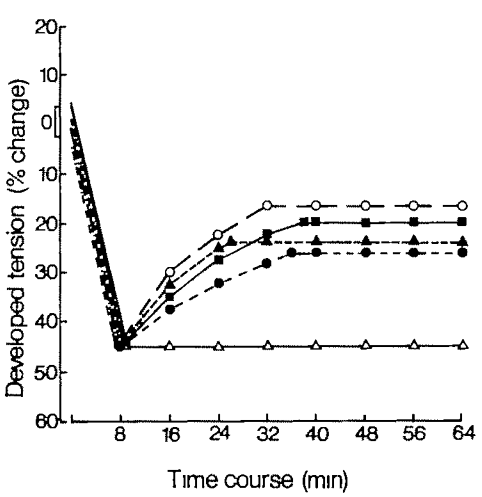


Figure 2 The developed tension (d*T*) of the isolated perfused hearts of guinea-pigs on changing the perfusion medium from normal Krebs-Henseleit to one with increased potassium (x 1.5) and the effect of adding digitoxin or its metabolites (all 0.1 μM) 8 min later The flow rate and temperature were as in Figure 1. (△) Control curve for Krebs-Henseleit buffer with increased potassium; (○) digitoxin; (●) digitoxigenin bis-digitoxoside; (■) digitoxigenin mono-digitoxoside, (▲) digitoxigenin. Each plot denotes mean value from 4 hearts.

developed tension. This occurred within 8 min, after which the new plateau was maintained (Figure 2). At the end of 64 min, the 8 min wash-out with normal K-H restored the d*T* to control levels promptly in all experiments (not illustrated). The plateau obtained during the perfusion of high potassium K-H served as a measure to evaluate changes in d*T* induced by the glycoside in its presence. Inotropic responses were observed with all four glycosides, digitoxin producing the greatest increase and the bis compound the least (Figure 2).

In the presence of increased potassium in the perfusion medium, the uptake by the whole heart was decreased by 20–30% for each glycoside and the uptake by the microsomal fraction was decreased by 40–50% (Table 2). The highest concentrations were again found in the microsomal fractions and again the maximal uptake was seen with digitoxin and there was no distinct difference among the metabolites.

Discussion

These experiments show that the cleavage products of digitoxin, that have lost one, two or three of the digitoxose residues, are active positive inotropic agents. Quantitatively, digitoxin itself had the greatest effect at the one concentration determined. Although some metabolism of digitoxin has been claimed to take place in heart tissue (Sjoerdsma & Fischer, 1951) most studies emphasize that glycosides are not rapidly

Table 1 The uptake and subcellular distribution of digitoxigenin glycosides perfused in normal Krebs-Henseleit medium through isolated hearts of guinea-pigs

	Homogenate		*Subcellular fractions (pmol/mg protein)*			
Drug (All 0.1 μM)	*pmol/g heart*	*pmol/mg protein*	*Nuclear*	*Mitochondrial*	*Microsomal*	*Supernate/ pellet ratio*
[^{3}H]-Digitoxin	576 48 ± 8.83	3.751 ± 0 673	1.640 ± 0.262	2 305 ± 0.283	2.626 ± 0.110	0.775 ± 0.064
[^{3}H]-Digitoxigenin bis-digitoxoside	335.78 ± 11.72	2.264 ± 0.466	0 415 ± 0 073	1.190 ± 0.061	1.385 ± 0.190	0.631 ± 0 043
[^{3}H]-Digitoxigenin mono-digitoxoside	323.47 ± 7 06	2.293 ± 0.532	0.436 ± 0.071	1.274 ± 0.082	1.527 ± 0.160	0.504 ± 0.061
[^{3}H]-Digitoxigenin	305.42 ± 3.76	2.235 ± 0.379	0.832 ± 0.053	1.077 ± 0.081	1.377 ± 0 059	0.686 ± 0.021

Each value: mean ± s.e. of 4 hearts.

or extensively transformed in the heart (Marcus, Pavlovich, Burkhalter & Cuccia, 1967; Kuschinsky, Lahrtz, Lüllman & van Zweiten, 1967). We have administered each metabolite in its pure form directly to the heart and therefore the effects recorded represent those produced by the unchanged drug. The quantitative difference in their effects on inotropic response appears to follow a pattern. With decreasing number of digitoxose residues, the inotropic responses are progressively reduced. While the quantitative difference between the bis and mono compounds was not significant ($P > 0.05$), the differences between digitoxin and its cleavage products were significant ($P < 0.01$), as also were the differences between digitoxigenin and the other members of this group ($P < 0.01$).

Progressively diminishing effects with step-wise removal of the digitoxose residues of digitoxin were not found in previous studies either of inhibition of brain Na-K ATPase (Wilson, Sivitz & Hanna, 1970) or of pharmacological action. Thus Lage & Spratt (1966) showed a decrease in lethality following intravenous injection in mice (a digitalis resistant species) with each decrease in the number of sugar residues as far as the mono-digitoxoside but digitoxigenin was the most toxic of four agents. Furthermore in tests using guinea-pig isolated atria, Lüllman & Peters (1971) observed the greatest inotropic activity with the mono-digitoxoside and the least with digitoxigenin. Hence relative potencies appear to vary with the species and test situation.

The concentration of digitoxin attained in the ventricle was almost twice that of its metabolites, indicating that the third sugar residue promotes both the accumulation of the glycoside by the heart and the positive inotropic response. The relationship between uptake and inotropic response was anomalous for digitoxigenin, since its uptake was comparable to that of the bis and mono compounds, while its inotropic response was less. Neither the uptake nor the inotropic responses can be explained by the lipid solubility characteristics of this series of drugs. The results suggest an important contribution by the digitoxose moieties to interaction of digitalis with receptor or binding sites in heart muscle.

The decrease in digitoxigenin glycoside uptake in the presence of increased potassium is in keeping with the observations with other digitalis glycosides (Dutta *et al.*, 1968; Dutta & Marks, 1972). Potassium ions appeared to inhibit the uptake of each of the digitoxigenin glycosides to a membranous binding site. Nevertheless all four glycosides have shown a marked positive inotropic effect in the presence of elevated potassium (Figure 2) and this indicates that the increased $[K]_0$ does not interfere with the interaction of digitaloids with the inotropic receptor.

Thus, from the various standpoints of potassium effect on uptake, subcellular distribution as well as positive inotropic effectiveness, it is clear that the cleavage products of digitoxin behave as typical digitaloids, resembling their parent compound but with less biological activity. On a molar basis, the cardiac

Table 2 The uptake and subcellular distribution of digitoxigenin glycosides perfused in Krebs-Henseleit medium with elevated potassium concentration (K × 1.5) through isolated hearts of guinea-pigs

	Homogenate		*Subcellular fractions (pmol/mg protein)*			
Drug (All 0.1 μM)	*pmol/g heart*	*pmol/mg protein*	*Nuclear*	*Mitochondrial*	*Microsomal*	*Supernate/pellet ratio*
[³H]-Digitoxin	371.36 ± 7.62	2.413 ± 0.514	0.993 ± 0.043	1.194 ± 0.061	1.282 ± 0.041	0.601 ± 0.034
[³H]-Digitoxigenin bis-digitoxoside	177.97 ± 4.41	1.303 ± 0.403	0.704 ± 0.041	0.738 ± 0.063	0.850 ± 0.071	0.811 ± 0.052
[³H]-Digitoxigenin mono-digitoxoside	201.04 ± 4.76	1.424 ± 0.451	0.781 ± 0.027	0.793 ± 0.041	0.936 ± 0.044	0.709 ± 0.061
[³H]-Digitoxigenin	207.83 ± 3.97	1.401 ± 0.394	0.509 ± 0.044	0.746 ± 0.061	0.915 ± 0.032	0.684 ± 0.063

Each value: mean ± s.e. of 4 hearts.

uptakes of digitoxin and its cleavage products were approximately three times greater than and the supernatant pellet ratios were about twice those of ouabain or digoxin studied under the same conditions (Dutta *et al.*, 1968).

This work was supported by grant HE–07051 from the National Heart Institute, U.S.A. Some of these results were presented at the meeting of the American Society for Pharmacology and Experimental Therapeutics, Fall 1971, in Burlington, Vermont, U.S.A. Please send reprint requests to S.D.

References

DUTTA, S., GOSWAMI, S., LINDOWER, J.O & MARKS, B.H (1968). Subcellular distribution of digoxin-H^3 in isolated guinea pig and rat hearts. *J. Pharmac. exp. Ther.*, **159**, 324–332.

DUTTA, S. & MARKS, B.H. (1969). Factors that regulate ouabain-H^3 accumulation by the isolated guinea pig heart. *J. Pharmac. exp. Ther.*, **170**, 318–325.

DUTTA, S. & MARKS, B.H. (1972). Species and ionic influences on the accumulation of digitalis glycosides by isolated perfused hearts. *Br. J. Pharmac.*, **46**, 401–408.

GORNALL, A.G., BARDAWILL, C.T. & DAVID, A.M.M. (1949). Determination of serum proteins by means of the biuret reaction. *J. biol. Chem.*, **177**, 751–766.

KUSCHINSKY, K., LAHRTZ, H., LÜLLMANN, H & VAN ZWEITEN, P.A. (1967). Accumulation and release of ^3H digoxin by guinea pig heart muscle. *Br. J. Pharmac.*, **30**, 317–328.

LAGE, G.L. & SPRATT, J.L (1966). Structure-activity correlation of the lethality and central effects of selected cardiac glycosides. *J. Pharmac. exp. Ther.*, **152**, 501–508.

LAUTERBACH, F. & REPKE, K. (1960). Die fermentative Abspaltung von D-Digitoxose, D-Cymarose und L-Thevetose aus Herzglykosiden durch Leberschnitte. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **239**, 193–203.

LULLMANN, H. & PETERS, T. (1971). The cardiac activity of digitoxin metabolites. *Eur. J. Pharmac.*, **14**, 204–205.

MARCUS, F.I., PAVLOVICH, J., BURKHALTER, L. & CUCCIA, C. (1967). The metabolic rate of tritiated digoxin in the dog: a comparison of digitalis administration with or without a "loading dose". *J. Pharmac. exp. Ther.*, **156**, 548–556.

MODELL, W., KWIT, N.T., DAYRIT, C, SHANE, S.J. & GOLD, H. (1948). Comparison of digitalis glycosides with their genins in man. *Fed. Proc.*, **7**, 246.

REPKE, K. (1959a). Über Spaltung und Hydroxylierung von Digitoxin bei der Ratte. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **237**, 34–48.

REPKE, K. (1959b). Die Bis- und Mono-digitoxoside des Digitoxigenins und Digoxigenins. Metaboliten des Digitoxins. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **237**, 155–170.

REPKE, K. (1963). Metabolism of cardiac glycosides. Proceedings. *First International Pharmacology Meetings*. **3**, 47–73.

SJOERDSMA, A. & FISCHER, C.S. (1951). The fixation of radioactive digitoxin by isolated hearts. *Circulation*, **4**, 100–104.

STRAUB, W (1916). Chemischer Bau und pharmakologische Wirksamkeit in der Digitalisgruppe. *Biochem. Z.*, **75**, 133–144.

WALTHER, R. (1943). Über Wirkungsunterschiede in der Digitalisreihe. *Naunyn-Schmiedebergs Arch. Exp. Path. Pharmak.*, **201**, 611–644.

WILSON, W.E., SIVITZ, W.I. & HANNA, L.T. (1970). Inhibition of calf brain membranal sodium- and potassium-dependent adenosine triphosphatase by cardioactive steroids. A binding site model. *Mol. Pharmac.*, **6**, 449–459.

Br. J. Pharmac. (1976), **56**, 443–447

UPTAKE OF 5-HYDROXYTRYPTAMINE IN DIFFERENT PARTS OF THE BRAIN OF THE RABBIT AFTER INTRAVENTRICULAR INJECTION

R.C. DOW & I. LASZLO
MRC Brain Metabolism Unit, Department of Pharmacology,
University of Edinburgh, 1, George Square, Edinburgh, EH8 9JZ

1 The uptake of 5-hydroxytryptamine (5-HT) was investigated in different areas of the rabbit brain (anterior hypothalamus, the raphe, the region of the substantia nigra, several cortical areas and the medulla oblongata) after intraventricular injection in pargyline pretreated animals by the formaldehyde-induced histochemical fluorescence method.

2 The distribution of fluorescence showed that the uptake of 5-HT, after circulation in the cerebrospinal fluid, caused a general increase in intensity of green yellow to yellow background fluorescence. There was an increased fluorescence in the nerve terminals, but no uptake occurred either in the cell bodies of neurones or in the glial cells.

Introduction

During an investigation of the uptake of 5-hydroxytryptamine (5-HT) after intraventricular injection, an accumulation of 5-HT was found in the glial cells of the area postrema of the rabbit (Dow, Laszlo & Ritchie, 1973) by the formaldehyde-induced fluorescence method described by Falck & Owman (1965). Biochemical estimations in the same experiments also showed an increase of 5-HT concentration in different areas of the brain. It appeared interesting to study the cellular distribution of 5-HT in other areas of the brain in order to find out if it is a general characteristic of the glial cells in the brain to take up this monoamine.

In this study the distribution of 5-HT in different areas of the brain was examined by the histochemical fluorescence method after intraventricular injection.

Methods

Thirty-three rabbits (New Zealand, white, of either sex, weight between 2.5 and 3.4 kg) were used.

Treatment with drugs: different doses of 5-HT creatinine sulphate (20, 40, 100 µg base) were injected into the right lateral ventricle through a guide tube by the method of Moir & Dow (1970) in 0.1 ml 0.9% w/v NaCl solution (saline). Pargyline hydrochloride (Eutonyl) 200 mg/kg was injected intraperitoneally alone, or 5 to 6 h before the injection of 5-HT. In each experiment one rabbit was used as a control without drugs, and one for obtaining tissue samples without formaldehyde treatment for the non-specific fluorescence. The rabbits in the control groups did not have the guide tubes implanted, since it was found that the intraventricular injection of saline did not influence the fluorescence picture. Animals were killed by injecting air into an ear vein 30 min after 5-HT injection. Brains were quickly removed, and areas to be investigated were dissected at 0°C. Visualization of monoamines was performed by the method of Falck & Owman (1965). The procedure used in these experiments with fluorescence microscopy and microspectrophotofluorimetry was described by Laszlo (1974). The fluorescence of the sections was observed with a BG3 (Schott) primary and a 50 (Zeiss) secondary filter. Various shades of colours are described by the nomenclature used by Loveland (1970), so that Green fluorescence corresponds to a wavelength of 513, and Yellow to 577, whilst in between Green-Yellow is 560, yellowish Green is 545 and greenish Yellow 570 nm. Identification of the areas was achieved by toluidine blue staining of the adjacent sections with the help of the stereotaxic atlas of the brain of the rabbit (Monnier & Gangloff, 1961).

In the preliminary experiments for the location of fluorescence, Nissl staining by the gallocyanine chromalum method (Drury & Wallington, 1967) was used on the same section as prepared for the fluorescence by the procedure described by Falck & Owman (1965). While this method had been found to be satisfactory for the study of the cellular localization of fluorescence in the area postrema (Fuxe & Owman, 1965; Dow *et al.*, 1973), in other parts of the brain it gave variable or uncertain results. Similar findings were obtained by using thionin and cresyl fast violet (Drury & Wallington, 1967) and Giemsa staining (Gurr, 1956). The reason for these discrepancies was

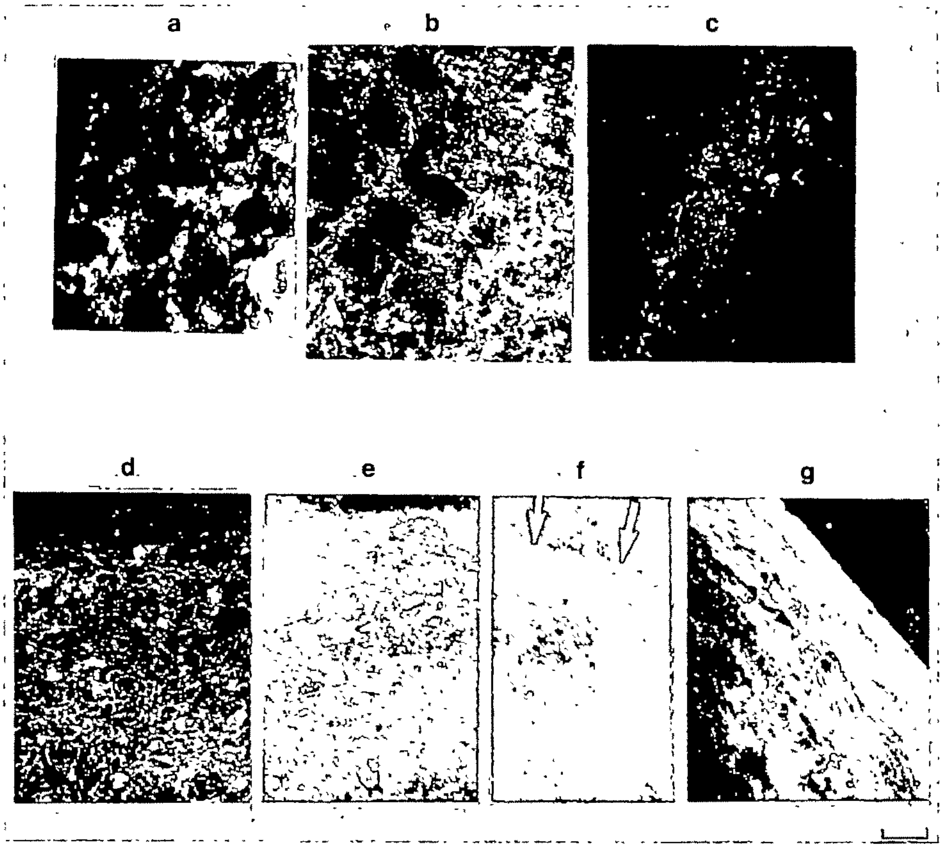


Figure 1 Fluorescence in various areas of the rabbit brain in control animals and after the intraventricular injection of various amounts of 5-hydroxytryptamine creatinine sulphate (5-HT) in animals given pargyline hydrochloride 5–6 h previously. (a) Nucleus supraopticus: control; (b) nucleus supraopticus. pargyline, 5-HT (100 μg); (c) nucleus suprachiasmaticus pargyline, 5-HT (40 μg); (d) lateral wall of the third ventricle: control; (e) lateral wall of the third ventricle: pargyline, 5-HT (100 μg), (f) lateral surface of the hypothalamus: control (arrows indicate the surface of the hypothalamus), (g) lateral surface of the hypothalamus: pargyline, 5-HT (40 μg). Calibration 20 μm.

thought to be the difference between the procedures used for preparing sections for fluorescence microscopy (Falck & Owman, 1965) and those which are used for Nissl staining (Drury & Wallington, 1967). An attempt was made to adopt the procedure for Nissl staining described by Drury & Wallington (1967) on the sections prepared for fluorescence microscopy by the method of Falck & Owman (1965), but the quality of staining did not improve essentially. Methods for staining glial cells, e.g. by Cajal's gold chloride sublimate method for astrocytes, Hortega's silver carbonate method for astrocytes, and Hortega's silver carbonate method for microglia (Drury & Wallington, 1967) were also tried on different animals, but none of these methods appeared to be applicable for sections prepared for fluorescence microscopy.

Since sections after fluorescence microscopy cannot be stained properly by any of the above mentioned methods, the cellular location of fluorescence was based on sections stained from different animals by the procedure described for Nissl staining by the gallocyanin chromalum method (Drury & Wallington, 1967).

Fluorescent sections were photographed on Ilford FP4, Din 22, with an exposure time from 45 to 60 s, or Ilford HP4 black and white films (Din 27–29) 15 to 60 s exposures.

Results

The fluorescence in the sections from the animals treated with different doses of 5-HT (20, 40 and

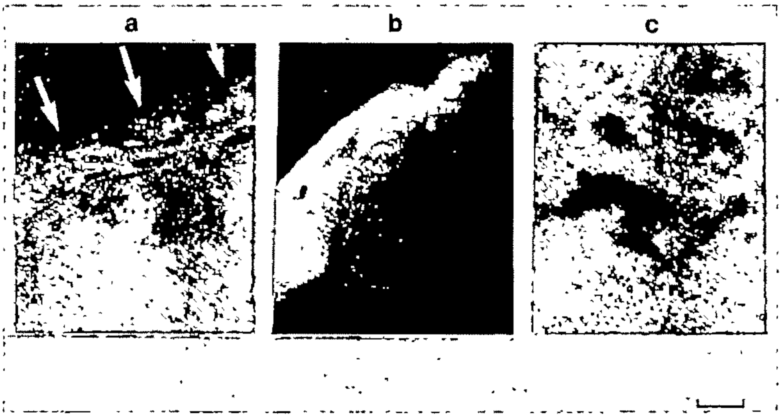


Figure 2 Fluorescence in various areas of the rabbit brain in control animals and after the intraventricular injection of various amounts of 5-hydroxytryptamine creatinine sulphate (5-HT) in animals given pargyline hydrochloride 5–6 h previously (a) Cortex (area postcentralis): control (arrows indicate the surface of the cortex); (b) cortex (area postcentralis): pargyline, 5-HT (40 μg); (c) cortex (area postcentralis): pargyline, 5-HT (40 μg) Calibration 20 μm

100 μg) was compared to that of the untreated (control) animals, and the following results were found.

Anterior hypothalamus: the Green background fluorescence of this area, including the chiasma opticum and the tractus opticus was more intense after 20 μg of 5-HT than in the control animals; after 40 and 100 μg, this difference was more marked, and the sections showed Green-Yellow fluorescence. The nerve endings in the whole area, especially in the nucleus supraopticus exhibited increased intensity of Green or Green-Yellow fluorescence after 20 μg of 5-HT. The nerve endings also appeared to be present in larger numbers, and the preterminals appeared elongated after 20 μg of 5-HT; after 40 and 100 μg, these differences were even more pronounced. In spite of the increased intensity of the fluorescence in the whole area, no uptake of 5-HT by cell bodies of neurones or glial cells was found (Figure 1a,b,c).

The ependyma of the third ventricle did not show fluorescence in either the sections from the control, or from the 5-HT-treated animals, although the area around the third ventricle exhibited increased intensity of fluorescence (Figure 1d,e) The surface of the brain, including the optic chiasma, did not exhibit fluorescence in control animals, but in the 5-HT-treated animals (20 μg) it showed increased intensity of yellowish Green fluorescence, which after higher doses (40 μg) of 5-HT became a fairly intense Green-Yellow or greenish-Yellow diffuse fluorescence layer (Figure 1f,g; Figure 2a,b,c). After 100 μg the colour in some cases showed Yellow, quickly fading fluorescence, as was found at the surface of the optic chiasma and in the meninges. The wall of the blood vessels, lateral to the nucleus supraopticus, after 100 μg of 5-HT also exhibited intense Yellow fluorescence. Estimates of intensity by microspectrophotofluorimetric method showed a similar distribution of fluorescence intensity to that described above.

The fluorescence picture in other parts of the brain, e.g. in the substantia nigra and in adjacent areas, in the raphe nuclei and in cortical areas as described by Falck, Mchedlishvili & Owman (1965), (e.g. the area postcentralis and insularis, the area occipitalis and temporalis, and the area occipitotemporalis) showed an essentially similar distribution of fluorescence. In these areas the background fluorescence after 20 μg of 5-HT (and pargyline) usually exhibited more intense Green or yellowish Green fluorescence than sections from control animals; after 40 μg the fluorescence became more intense Green-Yellow, and after 100 μg a greenish Yellow to Yellow very intense diffuse fluorescence was usually found. In spite of the generally increased intensity of background fluorescence, the cells remained dark in the whole area (Figure 2a,b,c).

After 5-HT-treatment the Green fluorescence, which is normally seen only at the terminals of nerve fibres in control animals, spread along the fibre so that they appeared more elongated. There was also more intense Green-Yellow fluorescence in the brain as well as around blood vessels at the surface of the brain.

The ependyma of the lateral ventricles and of the aqueduct in some cases after 5-HT showed a very quickly fading Yellow or Green fluorescent layer. In pargyline pretreated animals the surface of the brain and the areas near to it, after 20 μg of 5-HT exhibited

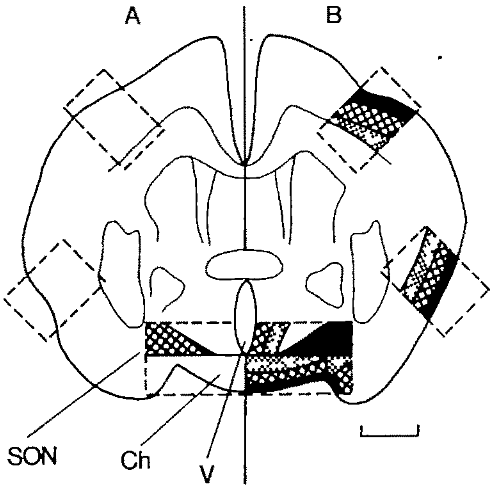


Figure 3 Schematic representation of the uptake of 5-hydroxytryptamine in different areas of the rabbit brain after intraventricular injection Frontal plane corresponding to the level of the area preoptica (redrawn after Monnier & Gangloff, 1961). SON = supraoptic nucleus; Ch = optic chiasm; V = third ventricle, A = control; B = 40 or 100 μg 5-hydroxytryptamine creatinine sulphate 5–6 h after pargyline. Calibration: 3 mm. Areas marked by – – – line indicate the regions investigated. Intensity of fluorescence: dotted areas = low; cross-hatched areas = medium; shaded areas = high intensity

Green or Green-Yellow diffuse fluorescence, and an intense fluorescent layer was usually present, corresponding to the meninges; after 40 and 100 μg the colour of fluorescence became greenish Yellow or Yellow. The fluorescence at the surface after the photographic exposure (1 min) became Green. After the intraventricular injection of 5-HT, a layer exhibiting an increased fluorescence, compared to the control, was found 1 to 2 mm from the external and internal surfaces of the brain (Figure 1d,e; Figure 2a,b). A schematic representation of the uptake of 5-HT can be seen in Figure 3 in the plane corresponding to the anterior hypothalamus. Similar distribution of the uptake was found in the region corresponding to the frontal planes of the raphe nuclei and the substantia nigra as well. Walls of blood vessels (at the surface of the brain) after 5-HT-treatment exhibited increased intensity of Green-Yellow fluorescence.

The fluorescence of the area postrema after 100 μg of 5-HT and pargyline was examined, and was found to be similar to that which had been described by Dow *et al.* (1973), showing increased number and intensity of Green fluorescent cells and Yellow fluorescent granules. No attempt has been made to characterize specifically the cell bodies showing fluorescence in the area postrema; it is probable from our earlier studies (Dow *et al.*, 1973) that they are glial cells. The ependyma of the central canal exhibited a very quickly fading fluorescent layer, which was more intense than in the control. Adjacent to the area postrema were present increased number of Green fluorescent varicosities, but there was no uptake of 5-HT by cell bodies.

The sections from animals injected only with pargyline showed an increased intensity of specific fluorescence, in agreement with Fuxe & Owman (1965), but this was less marked than after the intraventricular injection of 5-HT in pargyline pretreated animals.

Discussion

The earlier finding that glial cells in the area postrema of the rabbit contained 5-HT after intraventricular injection (Dow *et al.*, 1973) prompted a study of the uptake in other parts of the brain under the same experimental conditions. The areas of this study were selected on functional as well as morphological grounds, e.g. the anterior hypothalamus and the region of the raphe nuclei, which contained 5-HT nerve terminals and nerve cells, respectively (Carlsson, Falck & Hillarp, 1962; Fuxe, Hökfelt & Ungerstedt, 1969; Fuxe & Jonsson, 1974; Jonsson, Einarsson, Fuxe & Hallman, 1974); the cortical areas and the region of the substantia nigra, where the cell bodies of neurones and glial cells could be identified on the basis of morphological differences. This was necessary since identification by the conventional staining methods (e.g. Nissl staining or glial staining) on the sections for fluorescence microscopy did not appear satisfactory. The presence of freeze artefacts in cell bodies or freeze dried sections were mentioned by Dahlström & Fuxe (1964), who found that Nissl staining did not give satisfactory results on these sections.

The region adjacent to the area postrema also appeared interesting for this study, since the uptake of 5-HT by glial cells had been found previously in the area postrema (Dow *et al.*, 1973). The results obtained by the histochemical fluorescence method showed no uptake of 5-HT by cell bodies of neurones or glial cells in any of these parts of the brain, apart from the area postrema, which had been described earlier (Dow *et al.*, 1973). In contrast nerve endings, especially in the anterior hypothalamus, showed a marked uptake of this monoamine. An intense fluorescence was regularly found in the wall of the blood vessels after 5-HT treatment, as in the previous study by Dow *et al.* (1973). An accumulation of noradrenaline and 5-HT in arterial smooth muscle was also described by Buchan, Lewis & Sugrue (1974).

The colour of fluorescence after increasing doses of 5-HT changed toward the longer wavelengths; a shift of colour during photographic exposure in the opposite direction was also found in sections of 5-HT-treated animals. Both phenomena can be explained by the relationship between concentration and colour of fluorescence described by Laszlo (1975a, 1975b). The quickly fading fluorescence in the sections is characteristic of 5-HT (Falck & Owman, 1965).

The lack of uptake of 5-HT by the glial cells in different parts of the brain compared to the area postrema after its intraventricular injection can be explained by the characteristics of glial cells and those of the ependyma in these areas. The flat cell bodies of the ependyma over the area postrema differ from those of the rest of the brain (Wislocki & Putnam, 1920; Clemente & Breemen, 1955; Morest, 1960), and a free access of 5-HT could occur from the cerebrospinal fluid, where it is present in a high concentration after intraventricular injection. The glial cells in the area postrema also differ structurally from mature astrocytes in other parts of the brain (Wislocki & Putnam, 1920; Clemente & Breemen, 1955; Morest, 1960).

The uptake of 5-HT after intraventricular injection in pargyline pretreated rabbits produces greater intensity of fluorescence from both surfaces of the brain inward. Neither cell bodies of neurones nor glial cells appeared to contain this monoamine other than in the area postrema.

We are very grateful to Dr G.W. Ashcroft and to Dr D. Eccleston for the continuous help and discussion during this study, and to Dr G. Arbuthnott for his help in the preparation of the manuscript. We wish also to thank Mr I. Lennox for the illustration, and Abbot Laboratories Ltd., for the generous supply of pargyline hydrochloride (Eutonyl).

References

BUCHAN, P, LEWIS, A.J. & SUGRUE, M.F. (1974). A comparison of the accumulation of noradrenaline and 5-hydroxytryptamine into arterial smooth muscle. *Br. J. Pharmac.*, **52**, 132–133P.

CARLSSON, A., FALCK, B & HILLARP, N.-Å. (1962). Cellular localization of brain monoamines. *Acta physiol. scand.*, **56**, Suppl. 196.

CLEMENTE, C.D. & VAN BREEMEN, V.L. (1955). Nerve fibers in the area postrema of cat, rabbit, guinea pig and rat. *Anat. Rec.*, **123**, 65–79.

DAHLSTRÖM, A. & FUXE, K. (1964). Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta physiol. scand.*, **62**, Suppl. *232*.

DOW, R C., LASZLO, I & RITCHIE, I.M. (1973). Cellular localization of the uptake of 5-hydroxytryptamine in the area postrema of the rabbit after injection into a lateral ventricle. *Br. J. Pharmac.*, **49**, 580–587.

DRURY, R A B. & WALLINGTON, E.A. (1967). *Carleton's Histological Technique.* 4th edition. pp. 266–280. New York, Toronto: Oxford University Press.

FALCK, B, MCHEDLISHVILI, G.I. & OWMAN, Ch. (1965). Histochemical demonstration of adrenergic nerves in cortex-pia of rabbit. *Acta pharmac. tox.*, **23**, 133–142.

FALCK, B. & OWMAN, C. (1965). A detailed methodological description of the method for the cellular demonstration of biogenic monoamines. *Acta Univ. lund.* Sect. II. No. **7**, 1–23.

FUXE, K., HÖKFELT, T. & UNGERSTEDT, U (1969). Distribution of monoamines in the mammalian central nervous system by histochemical studies. In *Metabolism of Amines in the Brain.* Proceedings of a Symposium of the British and Scandinavian Pharmacological Societies. Edinburgh, July, 1968. ed. Hooper, G. pp. 10–22. London: McMillan and Co. Ltd.

FUXE, K. & JONSSON, G. (1974). Further mapping of central hydroxytryptamine neurons: studies with the neurotoxic dihydroxytryptamines. In *Advances in Biochemical Psychopharmacology.* Vol. 10, ed. Costa, E. & Greengard, P. pp. 1–12. New York: Raven Press.

FUXE, K. & OWMAN, C. (1965). Cellular localization of monoamines in the area postrema of certain mammals. *J. comp. Neurol.*, **125**, 337–354.

GURR, E. (1956). *A Practical Manual of Medical and Biological Staining Techniques,* p. 106. London: Leonard Hill (Books) Ltd.

JONSSON, G., EINARSSON, P, FUXE, K. & HALLMAN, H. (1974). Microspectrofluorimetric studies on central 5-hydroxytryptamine neurons. In *Advances in Biochemical Psychopharmacology.* Vol. 10, ed. Costa, E & Greengard, P. pp. 55–65. New York: Raven Press.

LASZLO, I. (1974). Procedure for the histochemical demonstration of noradrenaline and 5-hydroxytryptamine in the central nervous system using the formaldehyde induced fluorescence method. *Lab. Pract.*, **23**, 299–304.

LASZLO, I. (1975a). Relationship between colour and concentration of noradrenaline and 5-hydroxytryptamine in the formaldehyde induced fluorescence method. *Lab. Pract.*, **24**, 146–150.

LASZLO, I. (1975b). Change of colour of fluorescence during exposure to ultraviolet light and its effect on photomicrography. *Med. biol. Illust.*, **25**, 97–102.

LOVELAND, R.P. (1970). *Photomicrography.* vol. 1, p. 3. New York: John Wiley and Sons, Inc.

MOIR, A.T.B. & DOW, R.C. (1970). A simple method for allowing perfusion of cerebral ventricles of the conscious rabbit. *J. appl. Physiol.*, **28**, 528–529.

MONNIER, M. & GANGLOFF, H. (1961). *Rabbit Brain Research.* Vol. 1. Atlas for Stereotaxic Brain Research on the Conscious Rabbit. Amsterdam: Elsevier.

MOREST, D.K. (1960). A study of the structure of the area postrema with Golgi methods. *Am. J. Anat.*, **107**, 291–303.

WISLOCKI, G.B. & PUTNAM, T.J. (1920). Note on the anatomy of the areae postremae. *Anat. Rec.*, **19**, 281–287.

(Received September 17, 1975.
Revised December 22, 1975.)

Br. J. Pharmac. (1976), **56**, 449–455

THE RELATIONSHIP BETWEEN CALCIUM AND INCREASED SENSITIVITY OF RABBIT AORTAE FOUR HOURS AFTER RESERPINE

O. CARRIER, JR. & R.K. HESTER

Department of Pharmacology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284, U.S.A

1 Four hours after reserpine, rabbit aortic strips were supersensitive to acetylcholine, isoprenaline and noradrenaline. The threshold concentration of the drugs necessary to induce a response was less and the maximum tension developed by the tissues was greater than in control strips.

2 Reserpine-treatment potentiated the contractile responses to $CaCl_2$.

3 Reserpine-treatment resulted in an increase in calcium uptake and an increase in the slow component of $^{45}Ca^{2+}$ efflux.

4 After resperine-treatment, the rate of relaxation from a potassium-induced contraction was decreased.

5 It is concluded that reserpine-induced supersensitivity is related to an enhanced ability of the tissue to retain and utilize calcium.

Introduction

Non-specific supersensitivity of vascular smooth muscle observed 24 h after administration of reserpine may be related to changes in calcium mobility (Carrier & Jurevics, 1973). We have previously observed that rabbit isolated atria become more sensitive to calcium as little as 4 h after reserpine administration (Jurevics & Carrier, 1973), and comparable changes in vascular calcium homeostasis have been detected (Carrier, Whittington-Coleman, Matheny & Shibata, 1970). This raised the question whether there might be an acute change in vascular sensitivity 4 h after reserpine. The present study was designed to test this possibility and to further our knowledge of reserpine-induced postsynaptic supersensitivity.

The present paper includes characterization of the sensitivity, the tissue calcium changes, and the alterations in radioactive calcium movements, occurring in aortic tissue 4 h after reserpine-treatment of rabbits.

Methods

Tissue preparation and procedure

New Zealand white rabbits of either sex weighing approximately 1.5 kg were given 3 mg/kg reserpine (Serpasil, CIBA) intramuscularly 4 h before they were killed (treated) or were used as untreated controls. They were killed by a blow to the head and exsanguinated. A section of the thoracic aorta between the aortic arch and the diaphragm was removed and placed in oxygenated Ringer solution. Helical aortic strips were then prepared according to the method of Furchgott (1960). For contractile tension studies, aortic strips were divided into two equal segments which were approximately 3 mm in width and 33–35 mm in length. Ligatures were placed on both ends of the muscle segments, and subsequently mounted vertically on a glass rod and placed in a tissue-organ bath containing Ringer solution with a final volume of 20 ml. One end of the muscle segment was attached to a Grass FT-03 force-displacement transducer and isometric contractions were recorded on a Grass Model 7 Polygraph.

The muscle preparations were equilibrated for 90 min and maintained under a baseline tension of 2 g before exposing them to an agonist. During the equilibration period, the tissues were washed with fresh Ringer solution every 15 min to prevent toxic accumulation of waste products (Altura & Altura, 1970). The solutions, both in the chamber and those used for wash, were continuously oxygenated with a 95% O_2 and 5% CO_2 and maintained at a constant temperature of 37°C. In experiments involving La^{3+}, 100% O_2 was used as indicated.

At the end of the initial equilibration period, dose-response relationships were obtained for different agonists as well as for $CaCl_2$. Dose-response relationships were obtained by exposing aortic strips to cumulatively increasing concentrations of each respective agonist, without any subsequent washout of the bath chambers until the maximal contractile response was reached. Cumulative responses to $CaCl_2$ could only be obtained in a calcium-free depolarizing Ringer solution (Godfraind & Kaba, 1969; Jurevics & Carrier, 1973). Before each successive concentration of agonist was added, the aortic strips were allowed to reach a new steady-state tension. No more than one agonist was utilized in any one preparation. No more than one dose-response curve was obtained with any one preparation.

Several experiments were carried out to check the equality of tone between control and reserpine-treated aortic strips. After the initial equilibration period in normal Ringer solution, sodium nitrite (1 mM), a non-specific relaxant, was added. Both control and reserpine-treated aortic strips showed the same amount of relaxation, indicating similar levels of basal tone.

ED_{50} values were obtained by graphically calculating the concentration of an agonist producing 50% of its maximum response. Mean ED_{50} values were expressed as geometric means (Fleming, Westfall, De La Lande & Jehlett, 1972).

Tissue incubation solutions

Normal Ringer solution contained (mM): NaCl 154.0, KCl 5.4, $CaCl_2$ 2.4, $NaHCO_3$ 6.0 and dextrose, 11.0. Calcium-free Ringer solution was identical except that $CaCl_2$ was omitted (calcium contamination was below the detection limits of the Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer and therefore < 1 μM).

Zero-calcium, depolarizing Ringer solution contained (mM): NaCl 94.0, KCl 60.0, $NaHCO_3$ 6.0, dextrose 11.0 and ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA), 0.01 was added.

Calcium-free depolarizing Ringer solution was identical except that EGTA was omitted.

In $^{45}Ca^{2+}$ experiments, Tris(hydroxymethyl) amino-methane (5 mM) was added instead of $NaHCO_3$ to normal Ringer solution, together with $LaCl_3$ (10 mM) (van Breemen, Farinas, Casteels, Gerba, Wuytack & Deth, 1973).

In the depolarizing solutions, KCl was substituted for an equivalent amount of NaCl to maintain isotonicity of the solution. All solutions were made up in double-distilled deionized water with the final pH adjusted and maintained throughout the experiment at 7.4 by the addition of NaOH as necessary.

Stimulating agents

The stimulating agents used in these studies were calcium chloride, acetylcholine bromide (Nutritional Biochemicals Corp.), (–)-noradrenaline bitartrate (Sigma Chemical Co.) and (±)-isoprenaline hydrochloride (Winthrop Labs). Doses of agonists are expressed in terms of molarity. Calcium chloride was dissolved in double-distilled deionized water in a concentrated stock form. Acetylcholine, noradrenaline, and isoprenaline were prepared immediately before starting an experiment.

Calcium uptake studies

The method of van Breemen *et al.* (1973) was used in these studies. Aortic strips were incubated in Tris-buffered normal Ringer solution for 90 minutes. After this initial equilibration, the bathing solution was changed to a similar Ringer solution containing trace amounts of $^{45}Ca^{2+}$ (New England Nuclear Corp.). Following incubation in the radioactive solution for specific time periods (5, 10, 15 and 30 min) 10 mM $LaCl_3$ was added to the same solution. Exactly 3 min later, the solution was changed to a calcium-free Ringer solution containing Tris buffer (5 m) and $LaCl_3$ (10 mM) and subsequently maintained in this solution for 45 minutes. Tissues were then removed from the bath, blotted, weighed and digested in 1 ml of NCS (Nuclear Chicago Corp. solubilizing solution; Amersham/Searle Corp.) and kept overnight in an oven maintained at 50°C. On the following day, 14 ml of toluene scintillation fluid (100 ml of toluene, 400 mg 2,5-diphenyloxazole (PPO) and 5 mg of 1,4-bis 2-(5-phenyloxazolyl) benzene (POPOP)) was added to the digesting solution. Tissue $^{45}Ca^{2+}$ was counted in a Nuclear Chicago Corp. Unilux Model 3 Liquid Scintillation counter.

Calcium content was calculated using the following equation:

$$\text{Calcium content (mmol/kg wet wt.)} = \frac{\text{ct/min in muscle}}{\text{kg wet wt}} \cdot \frac{\text{mmol Ca}^{2+}\text{ in labelled medium}}{\text{ct/min in labelled medium}}$$

$^{45}Ca^{2+}$ efflux studies

Aortic strips were incubated in normal Ringer solution for 90 min and then incubated for 30 min in a normal Ringer solution containing $^{45}Ca^{2+}$ (5×10^{-5} ct min^{-1} ml^{-1}). After this period of incubation and uptake, the strips were rinsed in 4 tubes containing 5 ml of normal Ringer solution (unlabelled) within a total time period of 20 seconds. These strips were then transferred to successive tubes containing 5 ml of calcium-free Ringer solution containing Tris-buffer (5 mM) at designated time intervals for the next 60 minutes. At the end of the final washout period, the tissue was blotted with mineral-free filter paper for not more than 90 s, following which its wet weight was determined. The tissue was then digested and counted using the same procedure as in the previous uptake studies. One ml of each washout solution was dissolved in 15 ml of liquid scintillation fluid containing 40% ethanol and 60% toluene scintillation solution and counted.

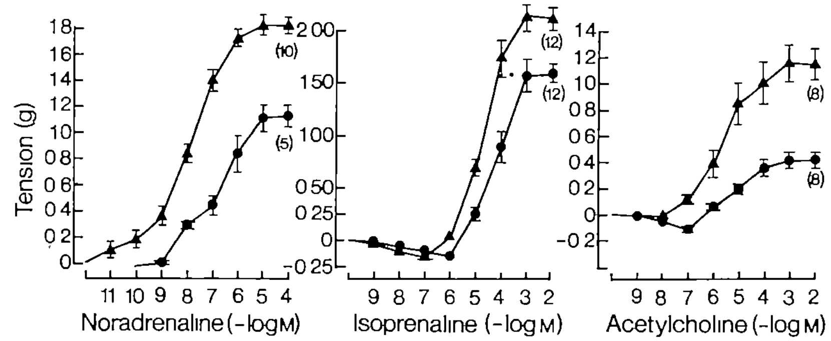


Figure 1 The responses of rabbit aortic strips from control animals (●) and from animals treated 4 h earlier with reserpine 3 mg/kg (▲) to cumulative concentrations of acetylcholine, isoprenaline and noradrenaline. Vertical bars represent s e mean. Numbers in parentheses are number of aortae used.

$^{45}\mathrm{Ca}^{2+}$ washout data were obtained by determining the $^{45}\mathrm{Ca}^{2+}$ present in each washout solution and the $^{45}\mathrm{Ca}^{2+}$ remaining in the tissue after the 60 min washout. Curves were then plotted which express the decline in tissue $^{45}\mathrm{Ca}^{2+}$ with time, indicating the amount of $^{45}\mathrm{Ca}^{2+}$ remaining in the tissue after each washout interval as a percentage of the initial tissue $^{45}\mathrm{Ca}^{2+}$ content (desaturation curves).

Statistical significance was determined by the use of Student's *t*-test. Differences were considered significant when $P < 0.05$.

Results

The influence of reserpine pretreatment on the contractile responses of rabbit aortic strips to noradrenaline, isoprenaline, and acetylcholine

The contractile responses to noradrenaline, isoprenaline and acetylcholine of aortic strips from reserpine-treated and untreated rabbits are illustrated in Figure 1. The maximum contractile responses for noradrenaline, isoprenaline, and acetylcholine were obtained at concentrations ⩾, 0.1 μM (10^{-5} M), 1 mM and 1 mM (10^{-3} M), respectively. The aortic strips from the reserpine-treated rabbits were supersensitive to all three agonists. The ED$_{50}$ for acetylcholine was 2.3 times, for isoprenaline 3.9 times, and for noradrenaline 18.1 times less for the aortic strips from reserpine-treated rabbits than for the strips from control rabbits. These differences were significant ($P < 0.02$). The threshold concentration for each agonist was significantly lower with the aortic strips from the reserpine-treated rabbits than with those from the untreated animals. For noradrenaline, all 5 control aortic strips had a threshold of 1 nM (10^{-9} M), while in the reserpine-treated aortic strips, 5 responded at 0.1 nM (10^{-10} M) and 5 at 0.01 nM (10^{-11} M). For isoprenaline, 10 control aortic strips had a threshold response at 10 μM (10^{-5} M) and 2 at 1 μM (10^{-6} M); while in reserpine-treated aortic strips 9 had a threshold at 1 μM (10^{-6} M) and 3 at 0.1 μM (10^{-7} M). For acetylcholine, the control threshold for all 8 of the aortic strips was 1 μM (10^{-6} M), while in the reserpine-treated group, 5 had a threshold response at 0.1 μM (10^{-7} M), and 3 at 0.01 μM (10^{-8} M). Also, with all three agonists, the maximum tensions developed by the aortic strips from the reserpine-treated rabbits were significantly greater than those of strips from untreated rabbits.

Reserpine had an interesting effect on the relaxant response of aortic strips to low doses of isoprenaline and acetylcholine. Strips from reserpine-treated rabbits consistently reached maximum relaxation at a lower concentration of isoprenaline than did aortic strips from untreated rabbits, while reserpine treatment abolished the relaxant phase of acetylcholine responses.

The influence of reserpine pretreatment on the contractile responses of rabbit aortic strips to calcium

Contractile responses to calcium can be induced when aortic strips are incubated in a calcium-free, depolarizing Ringer solution (Godfraind & Kaba, 1969; Carrier & Jurevics, 1973). The responses of rabbit aortic strips incubated in this media to increasing doses of calcium are illustrated in Figure 2. Although the ED$_{50}$'s for the strips from reserpine-treated and untreated rabbits were not different, all responses to calcium above the ED$_{50}$ level of the strips from reserpine-treated rabbits were significantly ($P < 0.01$) greater than those of the strips from the untreated rabbits. This became more pronounced as the concentration of calcium approached normal physiological values. The maximum contractile response of the aortic strips from reserpine-treated

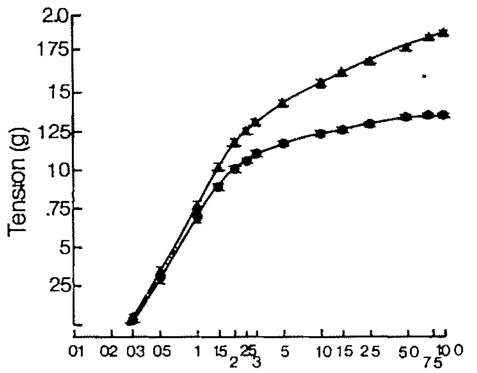


Figure 2 The responses of rabbit aortic strips from control animals (●) and from animals treated 4 h earlier with reserpine 3 mg/kg (▲) to cumulative concentrations of $CaCl_2$ in a calcium-free, depolarizing Ringer solution. Vertical bars represent s.e. mean. Number of aortae used was 12 for both control and reserpine-treated rabbits.

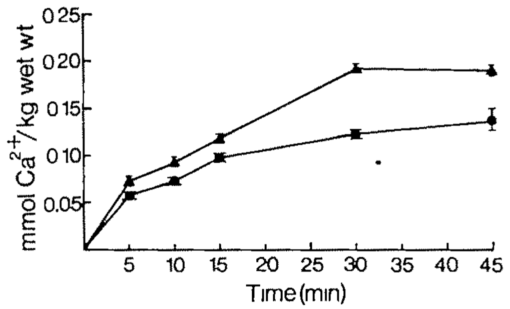


Figure 3 Uptake of calcium by rabbit aortic strips from control animals (●) and from animals treated 4 h earlier with reserpine 3 mg/kg (▲). Vertical bars represent s.e. mean. Number of aortae used was 6 for both control and reserpine-treated rabbits.

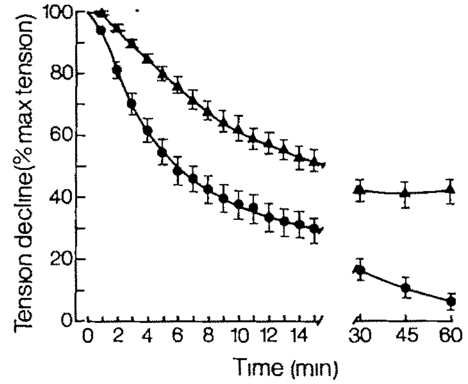


Figure 4 The decline in tension in a zero-calcium depolarizing Ringer solution of rabbit aortic strips from control animals (●) and from animals treated 4 h earlier with reserpine 3 mg/kg (▲) Vertical bars represent s.e. mean. Number of aortae used was 10 for both control and reserpine-treated rabbits.

rabbits (1.85 ± 0.01 g) was significantly ($P < 0.01$) greater than the responses (1.38 ± 0.03 g) of aortic strips from untreated rabbits.

The influence of resperine pretreatment on calcium uptake of rabbit aortic strips

The La^{3+} technique was utilized in order to determine the net transmembrane fluxes without interference from the extensive exchangeability of the calcium in the extracellular space and bound to external membranes. This method presumably defines cellular calcium, not only by eliminating extracellular calcium, but also by completely inhibiting further calcium influx, and almost completely inhibiting calcium efflux (70–90%) in rabbit aortic strips (van Breemen *et al.*, 1973; Freeman & Daniel, 1973). The accumulation of calcium by rabbit aortic strips, illustrated in Figure 3, was significantly greater at 5, 10, 15 and 30 min in the strips from reserpine-treated rabbits than in aortic strips from untreated rabbits. Both groups of aortic strips essentially attained maximum uptake at 30 minutes.

The influence of reserpine pretreatment on the tension decline of rabbit aortic strips in a zero-calcium, depolarizing Ringer solution

Aortic strips placed in a zero-calcium, depolarizing Ringer solution exhibited an initial contractile response which was not sustained. The tension of aortic strips from both reserpine-treated and untreated rabbits declined in 60 min to a new resting tension (Figure 4) after which there was no further significant decline. The control aortic strips developed 1.4 ± 0.1 g in 2.6 ± 0.1 min, while the treated aortic strips developed 1.5 ± 0.1 g in 2.3 ± 0.1 minutes. The tensions developed were not significantly different; however, the time to peak tension of the treated tissue was significantly less than that of the control group. The rate of decline in tension of the aortic strips from reserpine-treated rabbits was significantly less at all times than the rate of decline in tension of the strips from untreated animals. The time to decline to one-half maximum response was 7.3 ± 1.2 min for control aortic strips, and 25.6 ± 6.1 min for reserpine-treated strips ($P < 0.01$). In addition, the aortic strips from the

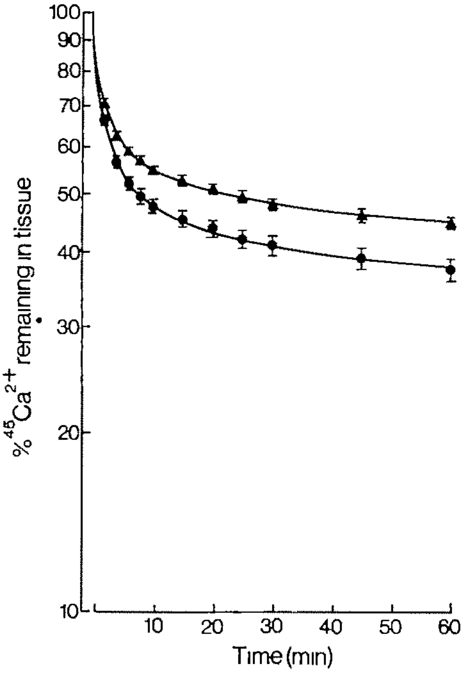


Figure 5 $^{45}Ca^{2+}$ efflux into calcium-free Ringer solution from rabbit aortic strips taken from control animals (●) and from animals treated 4 h earlier with reserpine 3 mg/kg (▲). The curves shown are desaturation curves, i.e. points represent $^{45}Ca^{2+}$ remaining in the tissue at the indicated time. Vertical bars represent s.e mean. Number of aortae used was 4 for both control and reserpine-treated rabbits.

reserpine-treated rabbits only declined to 42.0 ± 3.7% of their initial tension in 60 min, whereas the aortic strips from the untreated rabbits declined to 6.3 ± 2.7% of their initial tension in the same time period.

The influence of reserpine pretreatment on the $^{45}Ca^{2+}$ efflux from aortic strips

Since tension decline studies indicated that reserpine-treated tissues had an increased ability to maintain, or a decreased ability to remove, that ionized calcium pool needed to sustain contractile tension, studies of radioactive calcium efflux might give more insight into that calcium affected by reserpine pretreatment. Figure 5 illustrates the effect of reserpine pretreatment on the loss of $^{45}Ca^{2+}$ from aortic strips into a calcium-free Ringer solution after a 30 min equilibration period with the isotope. At the end of a 60 min washout period, control aortic strips still retained 37.2 ± 1.9% $^{45}Ca^{2+}$, while reserpine-treated aortic strips retained 44.2 ± 1.1% $^{45}Ca^{2+}$. The aortic strips from treated animals had a significant delay ($P < 0.01$) in $^{45}Ca^{2+}$ efflux from that of control strips at all points on the desaturation curve.

Discussion

The results obtained in the present study clearly show the development of a non-specific supersensitivity of rabbit aortic strips following a relatively acute treatment with reserpine. The dose-response curves for the three agonists, noradrenaline, isoprenaline, and acetylcholine, were shifted to the left and the threshold concentration of each was significantly decreased. The maximum tension obtained with each agonist was significantly increased. The significant decrease in the ED_{50} values is indicative of an increased sensitivity (Fleming, McPhillips & Westfall, 1973). It has been reported (Fleming & Trendelenburg, 1961; Hudgins & Fleming, 1966) that non-specific supersensitivity is usually associated with a slow time course of development with variations accompanying different methods, species and tissues. The present results clearly demonstrate that a non-specific supersensitivity can be acutely induced. Short-term development of supersensitivity characterized by similar changes has been also demonstrated in the heart (Jurevics & Carrier, 1973) and in the pineal gland (Romero & Axelrod, 1975). The possibility that this is a phenomenon different from the supersensitivity seen after 24 h or longer cannot be excluded.

There are many available theories explaining the mechanism of reserpine-induced supersensitivity. Hudgins & Fleming (1966) proposed that the change after reserpine-treatment of rabbits which results in supersensitivity occurs at a step beyond the level of receptor activation. Carrier & Shibata (1967) proposed that reserpine-treatment induced changes in essential ions (Na^+, K^+, and Ca^{2+}) leading to an increased cellular excitability and consequential supersensitivity. Many authors (Carrier & Shibata, 1967; Hudgins, 1969; Hudgins & Harris, 1970; Garrett & Carrier, 1971; Carrier & Jurevics, 1973) have been proponents of the involvement of the calcium ion in reserpine-induced supersensitivity. Fleming (1972) has demonstrated a partial depolarization of cell membranes of the vas deferens following treatment of guinea-pigs with reserpine and suggests it plays a role in supersensitivity. A partial depolarization was also reported for isolated hearts from reserpine-treated guinea-pigs (Taylor, Westfall & Fleming, 1974).

The concepts of a membrane depolarization and of electrolyte involvement in supersensitivity correlates well with the data obtained in these studies, for the increased calcium uptake by the reserpine-treated aortic strips essentially demonstrates an increase in the tissue calcium available for exchange. This might be explained by increased permeability of the membrane to calcium (Carrier & Shibata, 1967; Hudgins & Harris, 1970) as well as a partial membrane depolarization (Fleming, 1974) because a major portion of the measurable inward current which accompanies either depolarization or other increases in membrane permeability in smooth muscle is a result of calcium fluxes (Bohr, 1973). These data, in conjunction with the decrease in the rate of tension decline, and the apparent increase in the size of the slow-clearing component of $^{45}Ca^{2+}$ loss, indicate that the tissue has an increased exchangeable fraction and, in addition, has a greater ability to retain and maintain the calcium stores involved in contraction. Previous studies (Hudgins & Harris, 1970; Garrett & Carrier, 1971; Carrier & Jurevics, 1973) have shown that reserpine-induced supersensitivity is associated with an increased ability of the tissue to bind calcium. This may explain the increase in maximum tension seen in these studies.

The increase in sensitivity to different agonists helps to elucidate the possible mechanism of action of reserpine-induced supersensitivity. Carrier & Jurevics (1973) have demonstrated the dependence of acetylcholine's action upon extracellular calcium in supersensitive tissues. This is in agreement with the possibility of increased membrane excitability or permeability following reserpine treatment. Also, Triggle (1972) has shown that acetylcholine exerts its contractile stimulation by first mobilizing membrane bound calcium which is closely associated with the cholinoceptor. The increased sensitivity to noradrenaline and isoprenaline following reserpine treatment suggests reserpine has an effect on intracellular calcium stores, because these agonists activate contractile proteins by primarily mobilizing intracellular calcium (Seidel & Bohr, 1971; van Breemen *et al.*, 1973).

The data obtained in the present studies appear to indicate an increased ability of the reserpine-treated aortic strips to maintain the calcium pool essential to the contractile process. Essentially, we believe that reserpine acts as a 'pharmacological rectifier' in that it allows more calcium to be taken up due to an apparent increase in permeability and to enhanced calcium mobilization in response to these agonists, while concomitantly limiting or decreasing the amount that is actively sequestered and extruded.

This investigation was supported by AFOSR Grant No. 71-2074 and the National Institutes of Health Grant No. HL17899-01. This research is part of a dissertation thesis presented to the faculty of the Graduate Program of the University of Texas Health Science Center at San Antonio in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Pharmacology (R.K.H.).

References

ALTURA, B.M & ALTURA, B.T. (1970). Differential effects of substrate depletion on drug-induced contractions of rabbit aorta. *Am. J. Physiol.*, **219**, 1698–1705.

BOHR, D.F. (1973). Vascular smooth muscle updated. *Circulation Res.*, **32**, 665–672.

CARRIER, O., JR. & JUREVICS, H.A. (1973). The role of calcium in 'non-specific' supersensitivity of vascular muscle. *J. Pharmac. exp. Ther.*, **184**, 81–94.

CARRIER, O., JR. & SHIBATA, S (1967). A possible role for tissue calcium in reserpine supersensitivity. *J. Pharmac. exp. Ther.*, **155**, 42–59.

CARRIER, O., JR., WHITTINGTON-COLEMAN, P.J., MATHENY, J & SHIBATA, S. (1970). Tissue calcium loss following reserpine administration in rabbits. *Archs int. Pharmacodyn. Thér.*, **187**, 97–105.

FLEMING, W.W. (1972). Altered resting membrane potential (RMP) of supersensitive smooth muscle cells. Abstracts, *Fifth Intl. Cong. Pharmac.*, p. 70.

FLEMING, W.W. (1974). Electrophysiological correlations with postjunctional supersensitivity. *Fedn. Proc.* (In press).

FLEMING, W.W., MCPHILLIPS, J.J. & WESTFALL, D.P. (1973). Postjunctional supersensitivity and subsensitivity of excitable tissues to drugs. *Ergeb. Physiol.*, **68**, 55–119.

FLEMING, W.W. & TRENDELENBURG, U. (1961). The development of supersensitivity to norepinephrine after pretreatment with reserpine. *J. Pharmac. exp. Ther.*, **133**, 41–51.

FLEMING, W.W., WESTFALL, D.P., DE LA LANDE, I.S. & JEHLETT, L.B (1972). Log-normal distribution of equieffective doses of norepinephrine and acetylcholine in several tissues. *J. Pharmac. exp. Ther.*, **181**, 339–345.

FREEMAN, D.J. & DANIEL, E.E. (1973). Calcium movement in vascular smooth muscle and its detection using lanthanum as a tool. *Canad. J. Physiol. Pharmac.*, **51**, 900–913.

FURCHGOTT, R.F. (1960). Spiral-cut strips of rabbit aorta for *in vitro* studies of responses of arterial smooth muscle. *Methods Med. Res.*, **8**, 177–186.

GARRETT, R.L. & CARRIER, O., JR. (1971). Alteration of extracellular calcium dependence in vascular tissue by reserpine. *Eur. J. Pharmac.*, **13**, 306–311.

GODFRAIND, T. & KABA, A. (1969). Blockade or reversal of the contraction induced by calcium and adrenalin in depolarized arterial smooth muscle. *Br. J. Pharmac.*, **36**, 549–560.

HUDGINS, P.M. (1969). Some drug effects on calcium movements in aortic strips. *J. Pharmac. exp. Ther.*, **170**, 303–310.

HUDGINS, P.M. & FLEMING, W.W. (1966). A relatively non-specific supersensitivity in aortic strips resulting from pretreatment with reserpine. *J. Pharmac. exp. Ther.*, **153**, 70–80.

HUDGINS, P.M. & HARRIS, T.M. (1970). Further studies on the effects of reserpine pretreatment on rabbit aortas: Calcium and histologic studies. *J. Pharmac. exp. Ther.*, **175**, 609–618.

JUREVICS, H.A. & CARRIER, O., JR. (1973). The influence of reserpine on the calcium induced responses of spontaneously beating isolated rabbit atria. *J. Pharmac. exp. Ther.*, **184**, 189–197.

ROMERO, J.A. & AXELROD, J. (1975). Regulation of sensitivity to beta adrenergic stimulation in induction of pineal N-acetyltransferase. *Proc. Natl. Acad. Sci.*, **72**, 1661–1665.

SEIDEL, C.L. & BOHR, D.F. (1971). Calcium and vascular smooth muscle contraction. *Circ. Res.*, **28** & **29**, 118–1195.

TAYLOR, D.A., WESTFALL, D.P. & FLEMING, W.W (1974). Reserpine-induced changes in the transmembrane potential of atrial cells of the guinea-pig heart. *Fedn. Proc.*, p. 469.

TRIGGLE, D.J. (1972). Calcium, excitable membranes, and neurotransmitter action. *Progr. Surface Sci.*, **5**, 267–293.

VAN BREEMEN, C., FARINAS, B R., CASTEELS, R, GERBA, P., WUYTACK, F. & DETH, R (1973). Factors controlling cytoplasmic Ca^{++} concentration. *Phil. Trans. R. Soc. B.*, **265**, 57–71.

(Received August 19, 1975.
Revised December 16, 1975)

Br. J. Pharmac. (1976), **56**, 457–467

ISOTONIC AND ISOMETRIC RESPONSES OF DIFFERENT TONIC MUSCLES TO AGONISTS AND ANTAGONISTS

M.J. MICHELSON & S.A. SHELKOVNIKOV
Pharmacological Laboratory, Sechenov Institute of Evolutionary Physiology and Biochemistry, Leningrad, K-223, Academy of Sciences, USSR

1 With isotonic recording the percentage of muscle shortening as compared with the maximal possible shortening, and with isometric recording the percentage of developed tension were determined. In relatively 'thick' muscles, such as dorsal leech muscle, frog rectus abdominis or protractor pharynx of holothuria (0.3–0.8 mm thick), the concentrations of a full agonist (carbachol) producing a given percentage of tension, (e.g. 50%) are about 5 times greater than the concentrations, producing the same percentage of shortening. In 'thin' muscles the difference between the percentage of shortening and tension is either small (retractor dentis of the sea urchin, 0.1 mm thick, response to carbachol) or absent (guinea-pig ileum, 0.06 mm thick, responses to methylfurmethide). The possible mechanism underlying this difference is discussed.

2 With partial agonists (dodecamethonium and heptamethonium) the fractional tension of the frog rectus abdominis is always less than the fractional shortening and the correlation between shortening and tension is the same as in the case of full agonists.

3 The blocking activity of (+)-tubocurarine on the frog rectus abdominis is the same in isotonic and in isometric conditions.

4 On the frog rectus abdominis the alkylating agent, decamethonium mustard, does not produce any 'parallel shift' of the dose-response curve for carbachol, the only result of alkylation being a decrease in maximal response, which is more pronounced in isometric than in isotonic conditions. The degree of decrease is in accordance with the correlation between percentage of shortening and percentage of tension in the absence of alkylating agent. Probably this muscle does not possess any 'spare receptors'.

5 On the frog muscle the dose-isometric response curve for acetylcholine (ACh) is shifted toward greater concentration about 33-fold as compared with the dose-isotonic response curve but after the inhibition of cholinesterases the shift is only about 6-fold. The same shift (5-fold) is observed for carbachol, which is not hydrolysed by cholinesterases. The results with ACh are due to the fact, that after cholinesterase inhibition the sensitivity to ACh increases in isotonic conditions only 13-fold, but in isometric conditions it increases 71-fold. Probably under isometric conditions, when the muscle remains in the extended state, the rate of hydrolysis of ACh is much greater than under isotonic conditions when the muscle is shortened during contraction.

Introduction

The isotonic response (the shortening of the muscle during its contraction) and the isometric response (the tension developed during the contraction) coincide in some muscles, but differ greatly in other muscles. Clark (1926) compared the percentage of shortening of the frog rectus abdominis muscle produced by different concentrations of acetylcholine (ACh) under isotonic conditions and the percentage of tension developed under isometric conditions, taking as 100% the maximal shortening and the maximal tension of which the tissue was capable. He found that with shortening by 50% the tension developed was negligible (Figure 1). On the other hand Colquhoun & Tattersall (1970) observed no difference between the percentage of shortening and tension of the guinea-pig ileum longitudinal muscle in response to histamine.

Some physiological observations have also been published. For example indirect stimulation of tonic fibres of frog gastrocnemius muscle in isotonic conditions produced a strong tonic shortening but the tension was less than 50 g, whereas the maximal tension under isometric conditions was about 300 g (Vereshchagin, Zhukov & Leushina, 1950). Csapo (1954; 1960) studied the contractions of rabbit uterus segments elicited by electrical stimulation and showed that with practically maximal (100%) shortening the tension was only 5%.

In the present work we tried to determine why the

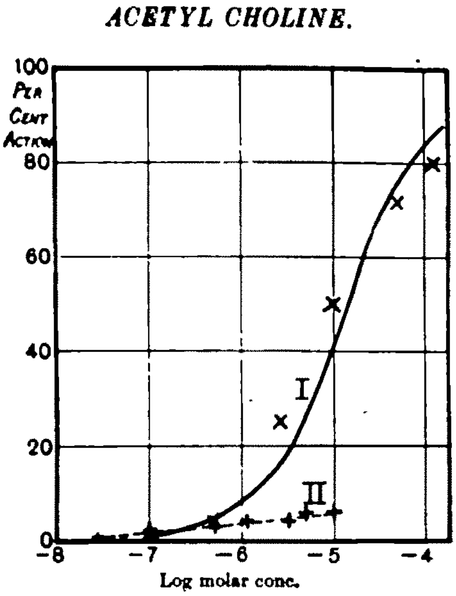


Figure 1 Action of acetylcholine on rectus abdominis as a function of concentration. I-isotonic, II-isometric. Ordinate scale: percentage of maximal possible action. Abscissa scale· logarithm of molecular concentration. From A.J. Clark (1926), *J. Physiol*, **61**, 530–546. By permission of *The Journal of Physiology*

correlation between shortening and tension is so different in different muscles. We also considered the importance of isotonic and isometric conditions when determining: (1) the EC_{50} values; (2) the capacity of drugs to produce a maximal contraction, showing whether it is a full or a partial agonist; (3) the blocking effect of antagonists; (4) the capacity of alkylating agents to bind covalently to the receptors; (5) the effect of anticholinesterases.

Methods

Frog rectus abdominis muscle (Rana temporaria)

A preparation of two unseparated muscles (left and right) was suspended in a bath, containing 25 ml of Ringer solution bubbled with air. For isometric recording the upper end of the muscle was connected to a tensoregister and the muscle was extended to its normal length. Even with maximal tension the muscle shortened only by 0.1 mm, that is no more than 0.2% of its length. With isotonic recording the load was 5–10% of maximal tension, that is about 1.5 g, the maximal tension being about 20 grams. In some experiments isometric responses were obtained first, and then isotonic responses, in other experiments the sequence was reversed. The response to isotonic KCl solution both in isotonic and isometric conditions was taken as 100%. The drugs were added to the bath in a volume not exceeding 0.5 ml. The interval between complete relaxation and the next contraction was 20 minutes.

Leech dorsal muscle (Hirudo medicinalis)

A piece of muscle containing 14 segments was used and the muscle was thoroughly cleaned of any traces of internal organs or nerve elements. Recordings were made as with frog muscle. In isotonic conditions the load was 1 g, the maximal tension being about 15 grams.

The pharynx protractor muscle of Holothuria (Cucumaria japonica)

This muscle was studied at the Putiatin Island Biostation in the Sea of Japan near Vladivostok. The animals were obtained by trawling and stored in a fish-pond in the sea. After excision the muscle was allowed to relax for 2 h in sea water with a load of 0.5 g and extended to its normal length of about 25 mm; this length was taken as 100%. The maximal tension developed by the muscle was 10–13 grams.

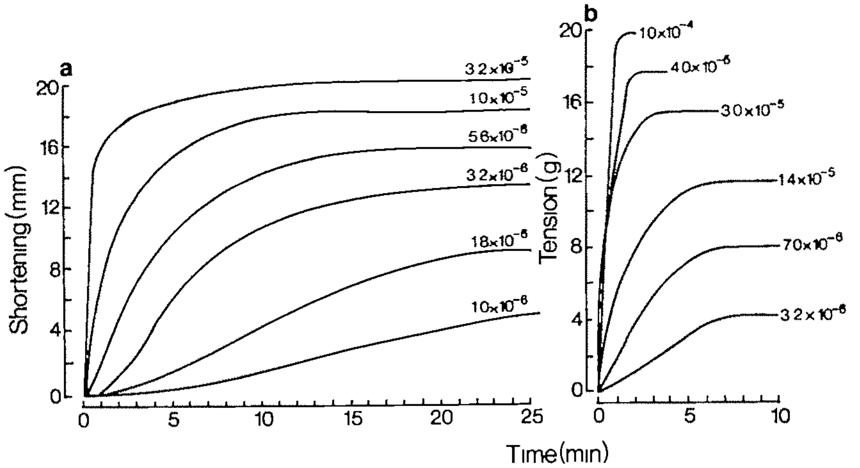
The retractor dentis muscle of the sea urchin (Strongylocentrotus droebachiensis)

This muscle was also studied at the Putiatin Biostation. With isotonic recording the load was 0.5 g; the maximal tension developed by the muscle was about 10 grams.

Guinea-pig ileum

A piece of guinea-pig ileum 5 cm long was suspended in a bath containing 5 ml Tyrode solution (bubbled with air) at pH 7.4 and 37°C. Hexamethonium (50 µg/ml) was used to block the nicotinic cholinoceptors of intestinal ganglia. The load in isotonic conditions was 400 mg, the maximal tension being about 6 grams. The drugs were added to the bath in a volume of 0.1 ml.

The full agonist used for all muscles was carbaminoylcholine (carbachol); on the frog muscle ACh was also used; on the guinea-pig ileum methylfurmethide was used. Dose-response curves were plotted from the results and the values for EC_{50} were calculated, that is, the concentration producing shortening or tension which was half of the maximum of which the tissue was capable. Heptamethylene-bis-trimethylammonium (BTM-7) and dodecamethylene-bis-trimethylammonium (BTM-12) were used as partial agonists on the frog rectus muscle. (+)-



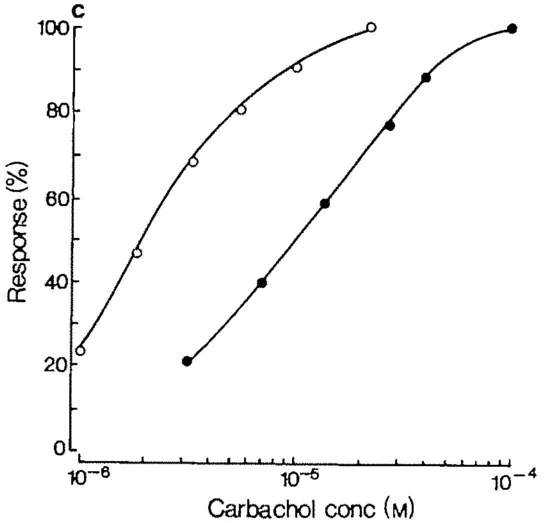
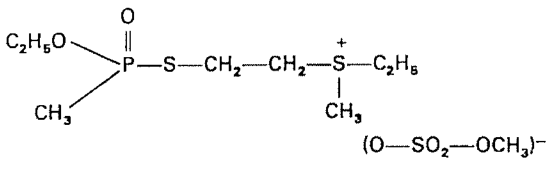


Figure 2 Frog rectus abdominis isotonic shortening (a) and isometric tension (b) of the same muscle, produced by different concentrations of carbachol. (c) Isotonic and isometric dose-response curves obtained in the same experiment: (○) isotonic shortening; (●) isometric tension. Both shortening and tension as % of maximum.

Tubocurarine was added to the bath for an hour and then the dose-response curve for carbachol was obtained and compared with the similar curve obtained without tubocurarine, both in isotonic and isometric conditions.

The irreversible organophosphorus cholinesterase inhibitor Gd-42 3×10^{-7} M was added to the bath containing the frog muscle for 30 min and then washed out for an hour. Isotonic and isometric dose-

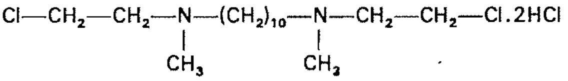


$$(C_2H_5O)(CH_3)P(=O)-S-CH_2-CH_2-\overset{+}{S}(CH_3)-C_2H_5 \quad (O-SO_2-OCH_3)^-$$

Gd-42

response curves were obtained before and after cholinesterase inhibition.

An alkylating agent A7 (decamethonium mustard) synthesized by D.V. Ioffe in S.G. Kuznetsov's laboratory was used for the irreversible block of frog rectus nicotinic cholinoceptors. This compound when



$$Cl-CH_2-CH_2-\underset{CH_3}{\underset{|}{N}}-(CH_2)_{10}-\underset{CH_3}{\underset{|}{N}}-CH_2-CH_2-Cl.2HCl$$

Decamethonium-mustard

dissolved in water forms an ethyleniminium ion, which alkylates the cholinoceptors. The formation of covalent bonds makes the blocking action very prolonged in spite of repeated washing. A7 was dissolved in Ringer solution at pH 7.4 and left for an hour at room temperature for formation of ethyleniminium ion. This agent was added to the bath in a concentration of 1×10^{-5} M for 1 h, after which the alkylating agent was washed out for 15 min with Ringer solution containing sodium thiosulphate 5×10^{-2}M, and then with fresh Ringer solution for 45 minutes. Sodium thiosulphate reacts with the free ethyleniminium ion and binds those molecules of the alkylating agent which have not bound covalently to the receptor. The action of A7 can be prevented by adding sodium thiosulphate (see Gill & Rang, 1966). The dose-response curves were obtained before and after alkylation.

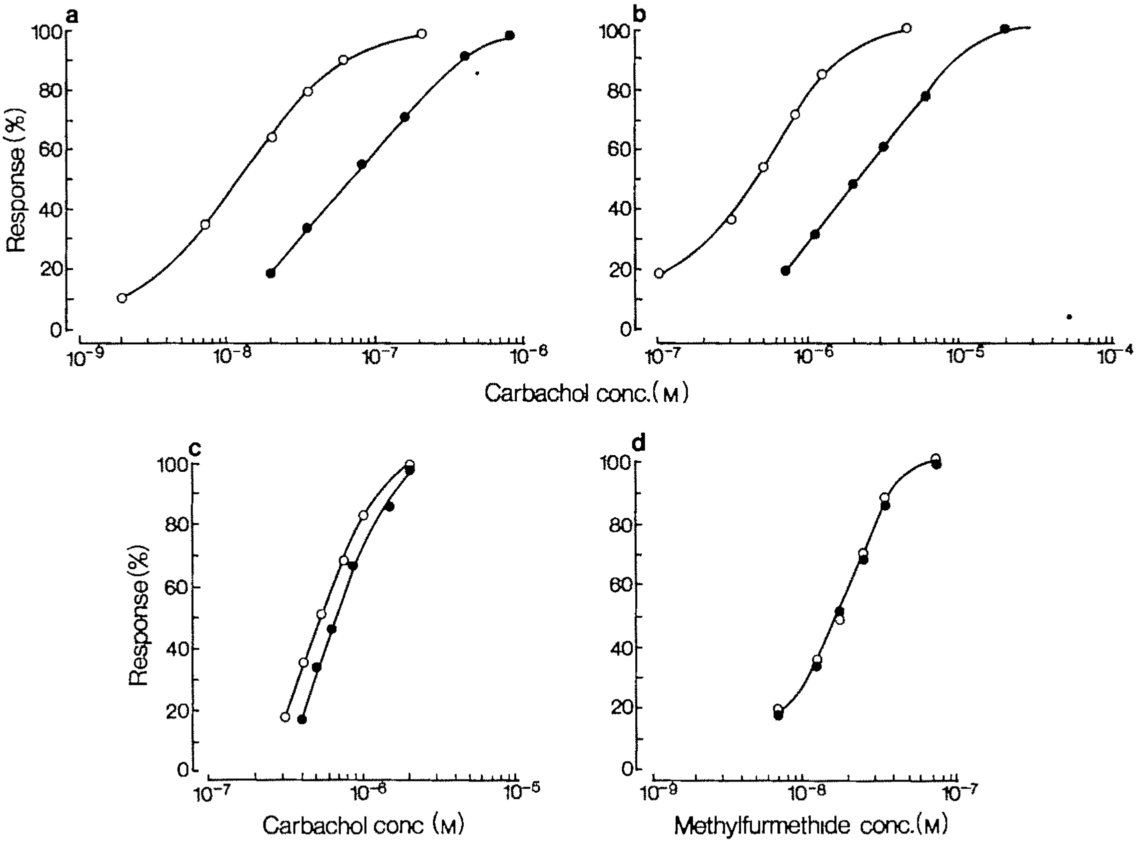


Figure 3 Isotonic and isometric dose-response curves, obtained on the same muscle. (○) isotonic shortening, (●) isometric tension, both as % of maximum (a) Protractor pharynx of holothuria, responses to carbachol; (b) leech dorsal muscle, responses to carbachol, (c) retractor dentis of the sea urchin, responses to carbachol; (d) guinea-pig ileum, responses to methylfurmethide

Results and Discussion

1. *The responses of different muscles to full agonists*

Frog rectus abdominus Figure 2 illustrates the isotonic (Figure 2a) and isometric (Figure 2b) responses of the frog rectus abdominis to different concentrations of carbachol. Comparison of the dose-response curves (Figure 2c) shows that a strong shortening corresponds to much less tension. For example, with a carbachol concentration producing 70% of maximal shortening only 20% of maximal tension is developed. On the other hand the carbachol concentration producing 50% tension can produce a shortening of about 90%. Table 1 shows that for this muscle the isometric EC_{50} is 5 times as great as the isotonic EC_{50}.

Holothurian pharynx protractor muscle The isotonic and isometric responses of this muscle (Figure 3a) are similar to those of frog muscle. For a given shortening in length the tension developed is much less; so with shortening by 80% the tension is only 30%. The isometric EC_{50} value is about 4.4 times as great as the isotonic value (Table 1).

Leech dorsal muscle With carbachol concentrations producing shortening of leech dorsal muscle by 80% the tension is 30% (Figure 3b). The isometric EC_{50} value is 4.3-times as great as the isotonic value (Table 1).

Sea urchin retractor dentis Both isotonic and isometric responses of the sea urchin retractor dentis develop much more rapidly than the responses of holothurian, leech or frog muscles. There is only a small discrepancy between the isotonic and the isometric dose-response curves (Figure 3c), but nevertheless the isometric curve is shifted towards greater concentrations. With shortening by 50% the tension is 40%. The isometric EC_{50} value is only 1.5 times as great as the isotonic value (Table 1).

Guinea-pig ileum In the guinea-pig ileum both isotonic and isometric responses develop very quickly, within a few seconds. The isotonic and isometric dose-response curves are practically superimposed (Figure 3d). A given methylfurmethide concentration produces the same percentage of shortening and of tension, hence the isotonic and isometric EC_{50} coincide (Table 1).

Thus on the frog, leech and holothurian muscles a given agonist concentration, that is the same stimulus, always produces less tension than shortening. The graphic relationship between shortening and tension has the form of a hyperbola (Figure 4). On the sea urchin muscle the difference is far less pronounced, but still the tension is less than the shortening. On the guinea-pig ileum there is no difference: any given concentration of the agonist produces the same percentage of shortening and of tension.

The different properties of the muscles studied can be compared with the differences in their thickness. Great differences between shortening and tension are observed in relatively thick muscles. The muscles of frog, holothuria and leech are 0.5, 0.8 and 0.3 mm thick respectively (Table 2). With the thin sea urchin muscle (0.1 mm) the discrepancy between shortening and tension is negligible. The guinea-pig ileum muscle is even thinner (0.06 mm) and the percentage of shortening coincides with the percentage of developed tension.

It is evident that a strong shortening of 'thick' muscles may be achieved when only a part of the muscle fibres is excited. With great shortening not all the fibres are shortened actively. If the fibres are parallel the active shortening of one part of the muscle fibres must be accompanied by passive folding of another part. But the passively folded fibres do not contribute to the muscle tension. Therefore a given agonist concentration may produce a considerable shortening and only a small tension.

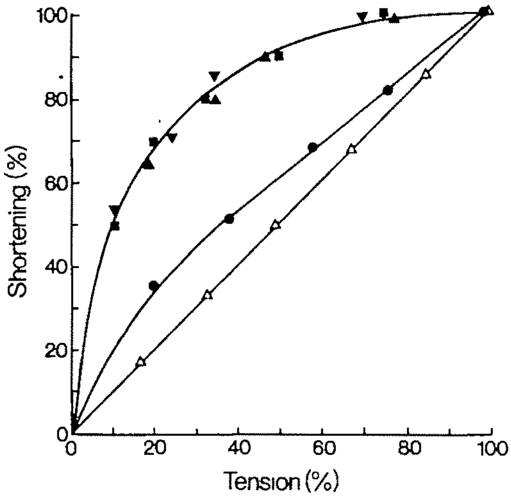


Figure 4 Relationships between shortening and tension (■) frog rectus abdominis; (▲) protractor pharynx of holothuria, (▼) leech dorsal muscle; (●) retractor dentis of the sea urchin; (△) guinea-pig ileum In all cases shortening and tension are expressed as % of maximum

One supposition that explains why only some fibres contract actively in the thick muscles is that in low concentrations the agonist cannot diffuse quickly into the deep internal layers of the muscle and only the superficial fibres are actively contracted, whereas the

Table 1 EC_{50} values for different muscles with isotonic and isometric recording

		EC_{50} (M)		*Ratio*
Muscle	*Agonist*	*Isotonic recording (mean ± s.e.)*	*Isometric recording (mean ± s.e.)*	*EC_{50} isometric / EC_{50} isotonic*
Frog rectus abdominis	Carbachol	$5.0 \times 10^{-6} \pm 0.7 \times 10^{-6}$ (10)	$2.6 \times 10^{-5} \pm 0.3 \times 10^{-5}$ (10)	5.0
Protractor pharynx of holothuria	Carbachol	$1.4 \times 10^{-8} \pm 0.2 \times 10^{-8}$ (15)	$6.1 \times 10^{-8} \pm 0.8 \times 10^{-8}$ (15)	4.4
Leech dorsal muscle	Carbachol	$4.0 \times 10^{-7} \pm 0.7 \times 10^{-7}$ (5)	$1.7 \times 10^{-6} \pm 0.14 \times 10^{-6}$ (5)	4.3
Retractor dentis of the sea urchin	Carbachol	$5.0 \times 10^{-7} \pm 0.6 \times 10^{-7}$ (4)	$7.0 \times 10^{-7} \pm 0.8 \times 10^{-7}$ (4)	1.4
Guinea-pig ileum	Methylfurmethide	$2.0 \times 10^{-8} \pm 0.3 \times 10^{-8}$ (10)	$2.0 \times 10^{-8} \pm 0.2 \times 10^{-8}$ (10)	1.0

The number of estimations is shown in parentheses deep fibres are passively folded (Figure 5a). This theory easily explains why in the quinea-pig ileum shortening coincides with tension: in a very thin muscle the agonist can act simultaneously on all fibres.

But this supposition may be correct only if the time of development and maintenance of the contracture is less than the time of diffusion of the agonist to the deep muscle fibres.

In Table 2 the time of development and maintenance of 50% shortening is compared with the time of diffusion of drugs into the deep layers of the muscles. To calculate the time of diffusion, that is the time of equalization of concentrations in the solution and in the deepest layers of muscle fibres, we used the diffusion equation $t=L^2/2D$, where t is the time in s, L = thickness of the muscle in cm and D = the diffusion coefficient (Setlow & Pollard, 1962). As we did not know the diffusion coefficient for carbachol, we used the diffusion coefficient for ACh determined on the rat diaphragm after inhibition of muscle cholinesterases, which is equal to 2.1×10^{-6} cm^2/s (Krnjević & Mitchell, 1960). The diffusion coefficients for the different drugs, ACh, decamethonium, gallamine, tubocurarine, are similar (Brookes & Mackay, 1971a, b).

One can see from the equation that the time of equalization of concentrations does not depend on the drug concentration, but only on the muscle thickness and diffusion coefficient. Hence the diffusion time will be the same with any agonist concentration in the bath. In the frog rectus, the holothurian and sea urchin muscles, and probably the leech muscle, the agonist can diffuse from both sides; therefore we used half the muscle thickness as the value of L. The thickness of the longitudinal muscle of guinea-pig ileum is not more than 60 μm (Paton & Rang, 1965). Probably the agonist can diffuse into this muscle only from one side.

Table 2 shows that the diffusion time is comparatively short in all muscles. The time of development and maintenance of 50% shortening is much greater, especially for the thick muscles. For instance the frog rectus muscle develops 50% shortening during 20 min and maintains it for hours whereas the diffusion into the muscle takes about 3.5 minutes. During the long time taken for the development and maintenance of the contraction the agonist has time to reach the deep fibres and contract them: in this case there should be no difference between the isotonic and isometric responses. These considerations therefore do not support the suggestion that the superficial fibres alone account for the isotonic response of thick muscles.

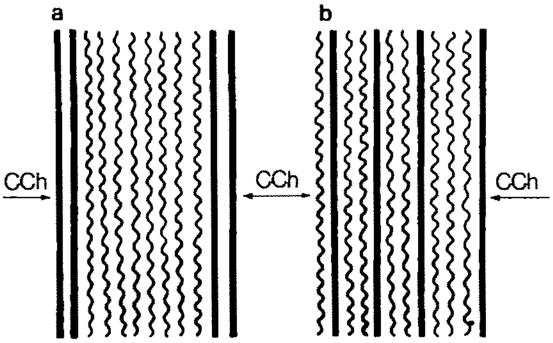


Figure 5 Two schemes of isotonic contraction of a thick muscle (a) Only superficial fibres are actively contracted (thick straight lines) and the deep fibres are passively folded (thin twisted lines) (b) Only the most sensitive fibres are actively contracted independently of their position in the muscle (thick straight lines) and the less sensitive fibres are passively folded (thin twisted lines).

Another suggestion is that in a thick muscle the most sensitive fibres contract first, independently of their position in the muscle. The shortening of these sensitive fibres is accompanied by a passive folding of less sensitive fibres and therefore the percentage of

Table 2 Muscle thickness, the time of diffusion and the time of development and maintenance of isotonic shortening

Muscle	*Thickness (cm)*	*Half thickness (cm)*	$t=L^2/2D$	*Time of development and maintenance of 50% shortening*
Frog rectus abdominis	0.05	0.025	approx. 200 s	>1 h
Protractor pharynx of holothuria	0.08	0.04	approx 400 s	approx. 1 h
Leech dorsal muscle	0.03	0.015	approx 60 s	>40 min
Retractor dentis of sea urchin	0.01	0.005	approx. 10 s	>5 min
Guinea-pig ileum	0.006	—	approx. 10 s	approx. 1 min

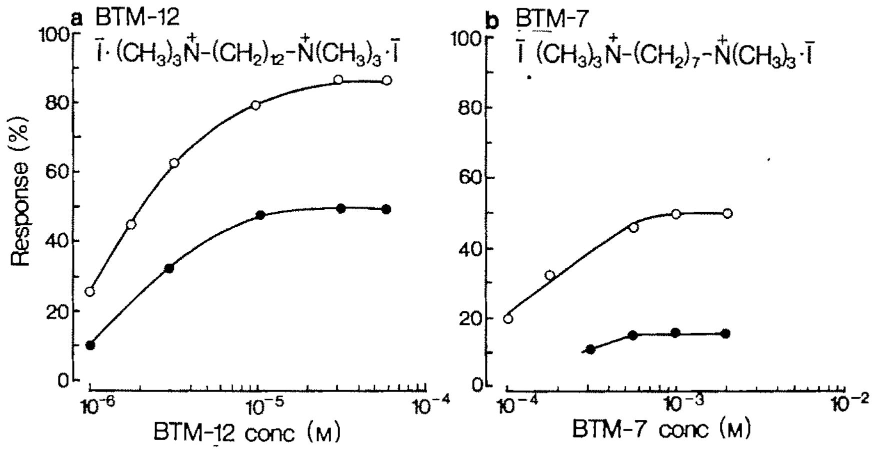


Figure 6 Frog rectus abdominis. Isotonic (○) and isometric (●) dose-response curves for partial agonists. (a) dodecamethylene-bis-trimethylammonium (BTM 12), (b) heptamethylene-bis-trimethylammonium (BTM 7) In both cases the isotonic and the isometric curves are obtained on the same muscle.

tension is always less than the percentage of shortening (Figure 5b). This suggestion seems more valid. It explains better the difference between the isotonic and isometric response of 'thick' muscles. It is more difficult to explain on this basis why isotonic and isometric responses in thin muscles are equal, because the fibres of a thin muscle may also differ in their sensitivity to an agonist. Possibly the conditions for folding of less sensitive fibres are better in a 'thick' than in a 'thin' muscle. The problem is still more complicated because the muscles studied have quite different structures.

2. *The action of partial agonists*

Each partial agonist (BTM-12 and BTM-7) was studied on five frog muscles. Figure 6a shows that the isometric dose-response curve is not only shifted towards greater concentrations as compared with the isotonic curve, but also that there is a more pronounced decrease in the maximal response. Thus the isotonic maximal response reaches 83% of shortening induced by KCl, whereas the isometric maximal response reaches 47% of the KCl-induced tension. In 5 muscles the mean isotonic maximal response was 85% ± 2%, and the mean isometric maximal response was 45% ± 2%. BTM-7 can produce only 50% shortening and the maximal tension developed is only 15% (Figure 6b). In 5 muscles the mean maximal isotonic response was 50% ± 3% and the mean maximal isometric response was 15% ± 1%.

These results are in accordance with the relationship between the isotonic shortening and isometric tension of frog rectus in response to a full agonist, carbachol. Thus if the relationship between isotonic and isometric responses to full agonists and the maximal response to a partial agonist with one mode of recording is known, it is possible to predict the maximal response with the other mode of recording.

3. *The blocking effect of (+)-tubocurarine*

On the frog rectus abdominis the isotonic and isometric responses to carbachol were obtained in the absence and in the presence of (+)-tubocurarine (2×10^{-6}M. In one series of experiments (4 muscles) the shift of isotonic responses towards greater concentrations of carbachol in the presence of tubocurarine was determined; in another series (5 muscles) the shift of isometric responses was studied. Figure 7 shows that the shift is equal in isotonic and in isometric conditions (Table 3). Such an equal shift could be predicted because the dose-response curves for carbachol are parallel with both methods of recording. Tubocurarine does not decrease either the isotonic or the isometric maximal responses; in both cases a maximal response can be obtained in the presence of tubocurarine provided the concentration of carbachol is sufficiently increased.

4. *The action of an alkylating agent A7*

On the frog rectus muscle the alkylating derivative of decamethonium, decamethonium-mustard A7, in a concentration of 1×10^{-5} M acts as a potent cholinolytic agent. A7 reduces the action of ACh, carbachol, suberyldicholine and tetramethylammonium, but does not influence the contracture produced by KCl. The action of A7 is very slowly reversible: the maximal response diminished by 50% is restored to 100% in 10 hours. Tubocurarine

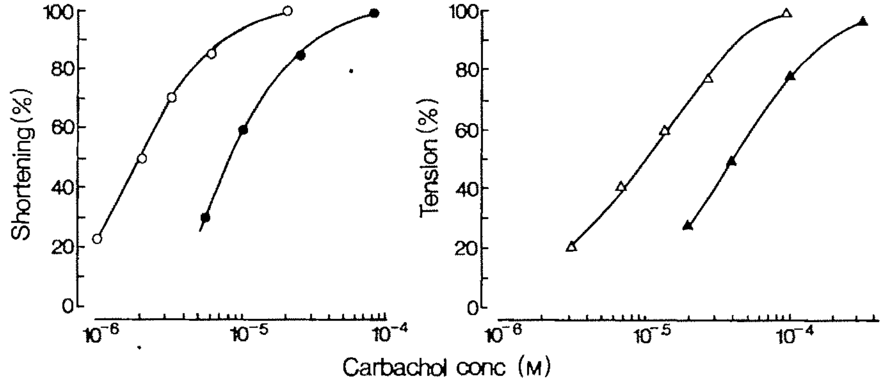


Figure 7 Frog rectus abdominis The parallel shift of dose-response curves for carbachol towards greater concentrations in presence of (+)-tubocurarine 2×10^{-6}M: (a) isotonic responses, (b) isometric responses In both cases the shift is about 4-fold. (● and ▲) In the presence; (O and △) in the absence of (+)-tubocurarine.

3×10^{-5}M (the concentration blocking 99% of cholinoceptors) provides full protection from A7. A7 produces a similar block of contractions induced by ACh, carbachol and tetramethylammonium and the change of pH from 6 to 11 does not influence the alkylating activity. It may be thought therefore that A7 is blocking the anionic site of the cholinoceptor, probably a carboxylic group the ionization of which hardly changes with changes of pH within these limits.

The action of A7 on isotonic responses was studied on 5 rectus muscles and on the isometric responses of another 5 muscles. In both cases A7 1×10^{-5} M was added for 60 minutes. Figure 8 shows that A7 produced a shift in the dose-response curves both for isotonic (Figure 8a) and isometric (Figure 8b) recording toward greater concentrations but the decrease of maximal tension was more pronounced than the decrease of maximal shortening. In 5 muscles the mean decrease of the isotonic response was to 82% ± 2% of maximal and the mean decrease of the isometric response was to 47% ± 1% of maximal. Figure 3 shows that without alkylating agents the shortening by 82% corresponds to a tension of 40%. Thus in muscles with a great difference between shortening and tension the effect of alkylating agents will also be different and, probably, the effect in isometric conditions is more informative.

Nickerson (1956) observed that after the action of an alkylating agent on the guinea-pig ileum the dose-response curve for histamine was shifted towards greater concentrations by two orders of magnitude. Nickerson concluded that only a small fraction of the receptor pool was necessary for production of maximal response, all others being 'spare' (or 'reserve') receptors which can be blocked without preventing the muscle from developing a maximal response provided the agonist concentration is sufficiently high. Waud (1968) doubted this interpretation because Nickerson recorded isotonic responses and administered the alkylating agent for a short time. Waud supposed that only superficial cells were blocked, but not the deep ones, and concluded that Nickerson was examining not spare receptors but spare cells. But on the guinea-pig ileum the isotonic

Table 3 Isotonic and isometric EC_{80} values for carbachol on the frog rectus abdominis in the presence and in the absence of (+)-tubocurarine (Tbc)

	EC_{80} (M)		Dose-ratio. *EC_{80} after Tbc* / *EC_{80} before Tbc*
Mode of recording	*before tubocurarine*	*in presence of tubocurarine* *2×10^{-6} M*	
Isotonic	$5.0 \times 10^{-6} \pm 0.8 \times 10^{-6}$ (4)	$2.1 \times 10^{-5} \pm 0.3 \times 10^{-5}$ (4)	4.2
Isometric	$2.5 \times 10^{-5} \pm 0.4 \times 10^{-5}$ (4)	$1.0 \times 10^{-4} \pm 0.1 \times 10^{-4}$ (4)	4.0

The number of experiments is shown in parentheses.

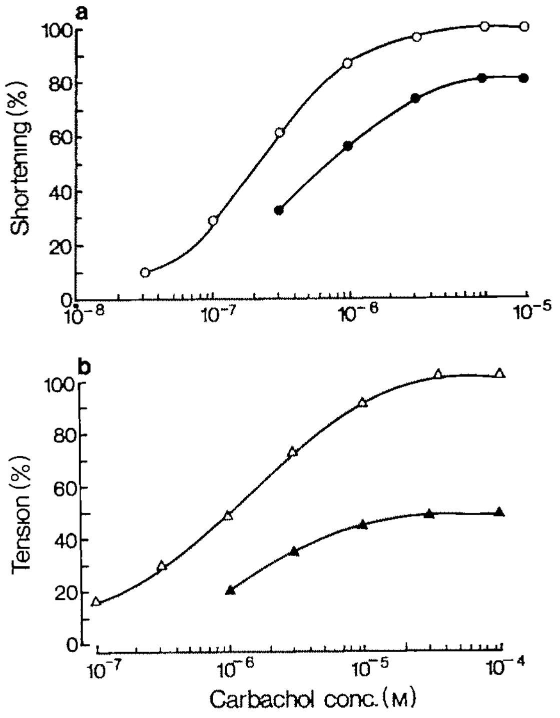


Figure 8 Frog rectus abdominis. The blockade of responses to carbachol after the action of the alkylating agent decamethonium-mustard (A7) 1×10^{-5}M during one hour (a) dose-isotonic response curves before (○) and after (●) the action of the alkylating agent, (b) dose-isometric response curves before (△) and after (▲) the action of the alkylating agent.

and isometric responses coincide, indicating that there are no spare cells. Indeed, we observed a similar action· of alkylating agents (benzilylcholine-mustard and others) on isotonic and isometric responses of guinea-pig ileum to methylfurmethide.

On the frog rectus 60% of tension corresponds to nearly 100% of shortening, hence there is no more than 40% of spare cells in this muscle. Thus after the action of an alkylating agent the isometric dose-response curve can be shifted toward greater concentrations (due to spare fibres) only 1.5-fold more than the isotonic curve. In our experiments on this muscle with A7 (1×10^{-5} M for 1 h) both isotonic and isometric responses to carbachol diminished at once and no parallel shift of dose-response curves was obtained. This invites the conclusion that there are no spare fibres and no spare receptors in this muscle.

5. *Isotonic and isometric responses of the frog rectus abdominis to acetylcholine: the effect of anticholinesterases*

The dose-isometric response curve for ACh is markedly shifted toward greater concentrations as compared with isotonic responses (Figure 9a), the mean dose-ratio being about 33 (Table 4) whereas for carbachol it is about 5. It seems probable that the greater dose-ratio in the case of ACh is due to the difference in the rate of its hydrolysis by the muscle in isotonic and in isometric conditions. To check this supposition we determined the dose-ratio for ACh after inhibition of muscle cholinesterases. The irreversible organophosphorus cholinesterase inhibitor Gd-42 in a concentration of 3×10^{-7} M was added to the bath for 30 min (this produces complete inhibition

Table 4 Isotonic and isometric responses of the frog rectus abdominis to acetylcholine before and after the inhibition of muscle cholinesterases by an organophosphorus inhibitor Gd-42

	EC_{50} (M)		*Dose-ratio.*
	before the inhibition of cholinesterases	*after the inhibition of cholinesterases*	*$\frac{EC_{50}\text{ before blockade}}{EC_{50}\text{ after blockade}}$*
Isotonic responses	$4.5 \times 10^{-6} \pm 0.5 \times 10^{-6}$ (10)	$3.5 \times 10^{-7} \pm 0.4 \times 10^{-7}$ (10)	13
Isometric responses	$1.5 \times 10^{-4} \pm 0.3 \times 10^{-4}$ (5)	$2.1 \times 10^{-6} \pm 0.2 \times 10^{-6}$ (5)	71
Dose-ratio· $\frac{\text{Isometric } EC_{50}}{\text{Isotonic } EC_{50}}$	33	6	

The number of experiments is shown in parentheses.

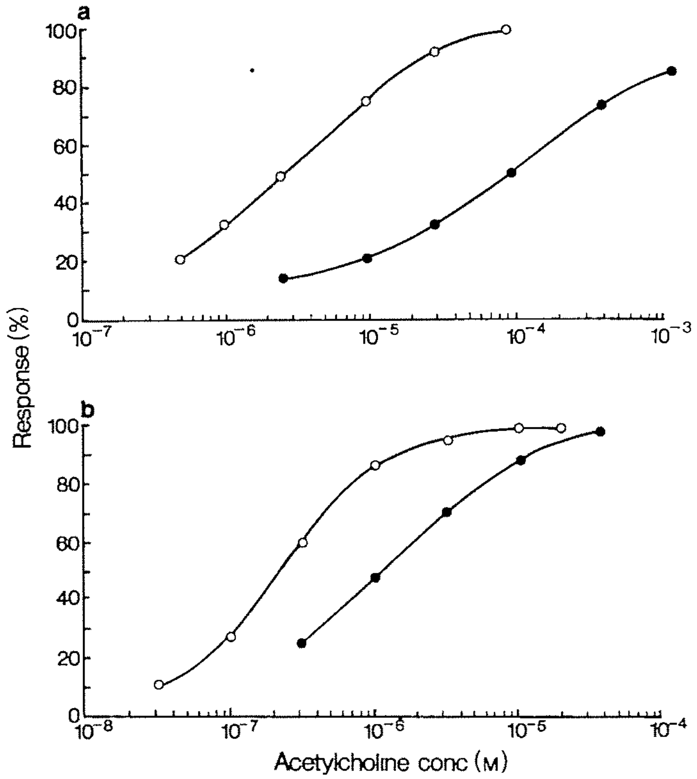


Figure 9 Frog rectus abdominis isotonic (○) and isometric (●) dose-response curves; (a) before exposure to the cholinesterase inhibitor; (b) after exposure to an irreversible cholinesterase inhibitor, Gd-42 3 × 10^{-7} M, for 30 minutes

of muscle cholinesterases), and then washed out for one hour. After this the mean dose-ratio of EC_{50} tension and EC_{50} shortening was not 33 but only about 6 (Table 4). After the inhibition of cholinesterases the isotonic dose-response curve was shifted 13-fold toward lesser concentrations, but the isometric dose-response curve was shifted 71-fold in the same direction (Table 4). Probably the rate of ACh hydrolysis by an extended muscle (isometric recording) is much greater than by a contracted (shortened) muscle (isotonic recording). Possibly the negligible increase of tension on the frog rectus muscle in response to ACh observed by Clark (1926) (see Figure 1) was due to the fact that in his experiments the muscle cholinesterases were not inhibited. Mittag, Ehrenpreis & Patrick (1971) have shown that the rate of ACh hydrolysis by an extended piece of guinea-pig ileum is much greater than by a contracted piece of ileum.

On the holothurian pharynx protractor without anticholinesterases we obtained similar results: the dose-ratio between isometric EC_{50} and isotonic EC_{50} was about 100. Unfortunately it was not possible to check this ratio after the inhibition of cholinesterases because the anticholinesterases induce spontaneous contractions of the muscle, but for carbachol the mean ratio was about 4.4 (Table 1).

Hence when studying agonists which are hydrolysed by cholinesterases it is important to take into account the fact that the rate of hydrolysis may be much greater with isometric recording than with recording under isotonic conditions.

We thank Dr Edith Bülbring for valuable criticism and help in the preparation of this manuscript.

References

BROOKES, N. & MACKAY, D. (1971a). Rate of onset and offset of neuromuscular block in the isolated rat diaphragm. *Br. J. Pharmac.*, **41**, 339–343.

BROOKES, N & MACKAY, D. (1971b). Diffusion of labelled substances through isolated rat diaphragm. *Br. J. Pharmac.*, **41**, 367–378.

CLARK, A J. (1926). The reaction between acetylcholine and muscle cells. *J. Physiol.*, **61**, 530–546.

COLQUHOUN, D. & TATTERSALL, M.L. (1970). Rapid histamine assays: a method and some theoretical considerations. *Br. J. Pharmac.*, **38**, 241–252.

CSAPO, A. (1954). Dependence of isometric tension and isotonic shortening of uterine muscle on temperature and on strength of stimulation. *Am. J. Physiol.*, **177**, 348–354.

CSAPO, A. (1960). Molecular structure and function of smooth muscle. In *The Structure and function of smooth muscle*, ed. Bourne, G.H., Vol. I, pp. 229–264. New York: Academic Press.

GILL, E.W. & RANG, H P. (1966). An alkylating derivative of benzilylcholine with specific and long-lasting parasympatholytic activity. *Mol. Pharmac.*, **2**, 284–297.

KRNJEVIĆ, K. & MITCHELL, J F. (1960). Diffusion of acetylcholine in agar cells and in the isolated rat diaphragm. *J. Physiol.*, **153**, 562–572.

MITTAG, T.W., EHRENPREIS, S. & PATRICK, P. (1971). Some properties of cholinesterases in intact guinea-pig ileum in vitro. *Arch. int. pharmacodyn.*, **191**, 270–278.

NICKERSON, M. (1956). On receptor occupancy and tissue response. *Nature, Lond.*, **178**, 697–698.

PATON, W.D.M. & RANG, H.P. (1965). The uptake of atropine and related drugs by intestinal smooth muscle of guinea-pig in relation to acetylcholine receptors. *Proc. Roy. Soc. B.*, **163**, 1–44.

SETLOW, R.B. & POLLARD, E C. (1962). *Molecular Biophysics*. Massachusets, USA, London, England: Addison-Wesley Inc.

VERESHCHAGIN, S.M., ZHUKOV, E.K. & LEUSHINA, L.I. (1950). The problem of the role of parabiotic excitation in the tonic contraction of striated muscle. *Physiolog. J. USSR*, **34**, 673–678. (In Russian.)

WAUD, D R. (1968). Pharmacological receptors. *Pharmac. Rev.*, **20**, 49–88.

(Received October 1, 1975.)

Br. J. Pharmac. (1976), **56**, 469–477

PROSTAGLANDIN PRODUCTION BY RABBIT ISOLATED JEJUNUM AND ITS RELATIONSHIP TO THE INHERENT TONE OF THE PREPARATION

S.H. FERREIRA[1], A.G. HERMAN[2] & J.R. VANE[3]
Department of Pharmacology, Institute of Basic Medical Sciences,
Royal College of Surgeons of England, Lincoln's Inn Fields, London WC2A 3PN

1 Pieces of rabbit jejunum were bathed in Krebs solution at 37°C in an isolated organ bath bubbled with O_2 and 5% CO_2. The bathing fluid was collected regularly and assayed for prostaglandins.

2 The preparations maintained a continuous sub-maximal muscle contraction, referred to as inherent 'tone'. Prostaglandins E_2 and $F_{2\alpha}$ were continuously generated by the intestine and released into the bathing fluid. The amounts released first declined over 2 h and then steadily increased. The release was also greater after 48 h storage in the refrigerator and after mechanical damage.

3 There was no change in prostaglandin release when the rabbit jejunum was contracted by acetylcholine or physostigmine or relaxed by adrenaline, hyoscine, papaverine, dinitrophenol, or calcium-free Krebs solution.

4 Addition to the bathing fluid of the prostaglandin precursor, arachidonic acid, did not increase the release of prostaglandins although it contracted the tissue. Thus, output of prostaglandins from the tissue was not limited by substrate concentration but more probably by the capacity of the prostaglandin synthetase.

5 Prostaglandin output was decreased by bubbling the bathing fluid with N_2 rather than O_2; at the same time the preparation relaxed.

6 Aspirin-like drugs such as indomethacin also decreased or abolished prostaglandin formation and this, too, was accompanied by loss of tone of the isolated preparation.

7 Pieces of rabbit jejunum stored in Krebs solution containing indomethacin initially released little or no prostaglandin into the bathing fluid. However, prostaglandin release increased with repeated washing of the preparation.

8 The results suggest that intra-mural prostaglandin production contributes to the inherent tone of the rabbit jejunum, that trauma increases prostaglandin production and that the inhibitory effects of anoxia are linked with the lack of prostaglandin production and activity. The relevance of these findings to intestinal activity *in vivo* is discussed.

Introduction

Some isolated smooth muscle preparations exhibit a sustained sub-maximal contraction or inherent 'tone', demonstrated by the fact that catecholamines and spasmolytic drugs cause relaxation. Weiland (1912) found a smooth muscle stimulant in the bath fluid in which segments of rabbit intestine were suspended. Similar stimulant activity, called 'Darmstoff', was obtained from frog isolated intestine (Vogt, 1949) and its diffusable component was later identified as a mixture of prostaglandins (Suzuki & Vogt, 1965). The spontaneous release of prostaglandins into the nutrient fluid by several other isolated preparations has since been described (Bennett, Friedmann & Vane, 1967; Coceani, Pace-Asciak, Volta & Wolfe, 1967; Vane & Williams, 1973). In general, tissues do not store prostaglandins so that release indicates fresh synthesis (Piper & Vane, 1971) and both synthesis and release are inhibited by non-steroidal anti-inflammatory drugs (Vane, 1971).

We have investigated the effects of indomethacin, a potent inhibitor of prostaglandin synthetase, on the generation of prostaglandins and on the tone of the rabbit isolated jejunum. We also investigated the effects on prostaglandin release of several other procedures which changed the tone of the isolated tissue. Some of these results have been presented to the British Pharmacological Society (Ferreira, Herman & Vane, 1972).

---

[1] Present address: Departamento de Farmacologia, Faculdade de Medicina, Ribeirao Preto, S.P.–Brazil.

[2] Present address: Universitaire Instelling Antwerpen, Department Farmaceutische Wetenschappen, Universiteitsplein, 1, 2610 Wilrijk, Belgium.

[3] Present address: Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS.

Methods

Rabbit isolated jejunum preparation

Rabbits (2–4 kg) were killed by exsanguination after a blow on the head. A segment (6–7 cm) of the proximal jejunum was removed and suspended in a 15 ml organ bath containing Krebs solution at 37°C and bubbled with 95% O_2 and 5% CO_2. In some experiments, N_2 was substituted for the O_2 and in others, the rabbit jejunum was superfused (Gaddum, 1953) with Krebs solution at 10 ml/min, rather than using an organ bath. The Krebs solution had the following composition in g/l (mM): NaCl 6.9 (118), KCl 0.35 (4.7), $CaCl_2.6H_2O$ 0.55 (2.5), KH_2PO_4 0.16 (1.2), $MgSO_4.7H_2O$ 0.29 (1.17), glucose 1.0 (5.6), $NaHCO_3$ 2.1 (25.0).

As well as the intact tissue, the following preparations were also used: (1) a strip of longitudinal smooth muscle prepared according to the method of Ambache (1954); (2) preparations in which the jejunum was everted, the mucosa damaged by scraping it with the sharp edge of a scalpel blade and then everted once more; (3) jejunum stored 48 h at 4°C in 50 ml Krebs solution with or without indomethacin (10 µg/ml).

Contractions of the muscles were detected by auxotonic levers (Paton, 1957) attached to Harvard Smooth Muscle Transducers. The bath fluid was left unchanged for periods of 30 min (collection period) and then drained for assay. In some experiments arachidonic acid was added to the bath fluid and after 30 min of incubation, the prostaglandin content of the fluid was compared with that found during the periods before adding and after removing the arachidonic acid.

Biological assay of prostaglandin-like material

Samples (1 ml) of the collected fluid were assayed on a bank of tissues consisting of a rat stomach strip, a rat colon and a chick rectum superfused in series with Krebs solution at 37°C at a rate of 10 ml/min to which a mixture of antagonists was added in order to make them specifically sensitive for detection and quantitation of prostaglandin-like material (Gilmore, Vane & Wyllie, 1968). The samples were assayed using prostaglandin E_2 as a standard and the results were expressed in terms of prostaglandin E_2 equivalents.

In the experiments in which physostigmine was used, the concentration of hyoscine was increased tenfold. When indomethacin or papaverine was added to the organ bath containing the rabbit jejunum, the same drug was also added to the Krebs solution superfusing the assay tissues (1 µg/ml for indomethacin and 4 µg/ml for papaverine). The results were analysed statistically by Student's *t*-test for paired or unpaired samples. Differences were considered significant if $P < 0.05$.

Destruction of prostaglandins by the rabbit isolated jejunum

Destruction of prostaglandins in the organ bath was studied by adding a known amount of prostaglandin E_2 to the bath fluid; after 30 min of incubation, the bath fluid was assayed and the amount of added prostaglandin E_2 recovered was calculated by subtracting the basal output of prostaglandins obtained during the 30 min collection period just before the addition of prostaglandin E_2, from the total amount assayed in the presence of the added prostaglandin E_2. The amount of added prostaglandin E_2 recovered after 30 min of incubation is expressed as a percentage of the activity added at the beginning of the incubation.

Chromatography

To confirm the presence of prostaglandins, bath fluid from scraped mucosa preparations was used: samples of consecutive collection periods were pooled, acidified with HCl to pH 3, extracted twice with ethyl acetate (1 : 1 v/v) and evaporated under reduced pressure. The residue was taken up in ethanol, spotted on a silica gel plate (F 254-Merck) and developed in the AI or AII system (Gréen & Samuelsson, 1964). The bands corresponding to prostaglandin E or prostaglandin F standards respectively were scraped off and the silica gel eluted with Krebs solution for bioassay. Intermediate bands were also assayed.

Drugs

The following drugs were used: arachidonic acid (Sigma), acetylcholine hydrochloride (BDH), adrenaline bitartrate (BDH), 2,4-dinitrophenol (BDH), physostigmine (eserine) sulphate (Hopkins & Williams Ltd), hyoscine hydrobromide (BDH), indomethacin (Merck, Sharp & Dohme), mepyramine maleate (May & Baker), methysergide bimaleate (Sandoz), noradrenaline bitartrate (Sigma), papaverine hydrochloride (Hopkins & Williams), phenoxybenzamine hydrochloride (SKF), propranolol hydrochloride (ICI), prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ (Upjohn). Concentrations of salts are expressed in terms of base.

Results

Basal output of prostaglandin-like material by different preparations of the rabbit isolated jejunum

Table 1 shows the basal output of prostaglandin-like material from different preparations at different time periods. The outputs from intact tissues were usually biphasic: the first sample tended to contain more activity than the third sample although these differences were not statistically significant. Thereafter, there was a gradual increase in output of activity and this continued for the rest of the experiment, so that after 4 h of incubation, the output was significantly higher than during the first 2 h ($P < 0.05$). In 2 experiments the jejunum was incubated for 8 h and the output was still increasing without reaching a plateau value.

The output from the scraped mucosa preparations was significantly higher than that from intact tissue ($P < 0.01$). This output was also biphasic: the 3rd or the 6th sample had significantly less activity ($P < 0.05$) than the first but then there was a gradual increase in release up to 8 hours.

Greater release was also seen when the tissues were stored for 48 h at 4°C as compared with the intact tissues ($P < 0.01$), and this release remained higher for the rest of the experiment. Addition of indomethacin to the Krebs solution during storage of the tissue prevented the initial increased release but the prostaglandin output rose steeply when the bath fluid was repeatedly changed and after 4–5 h reached values that were not significantly different from those of tissues stored without indomethacin.

In contrast to the progressive increase in output of prostaglandin-like material from these preparations, the output from the longitudinal muscle strip decreased with time, although the initial output was significantly greater than that from the intact tissues ($P < 0.01$).

Identification of the smooth muscle stimulating activity

In each of 9 experiments, extracts of the pooled bath fluid were chromatographed in the AI system. Activity was recovered in positions corresponding to F and E standards in a proportion of 1 : 1.01 ± 0.1 (mean ± s.d.). With the AII solvent system activity was only detectable in the E_2 and $F_{2\alpha}$ zones (4 experiments). No smooth muscle stimulating activity was found in other areas of the plates.

To find out how much of the rat stomach stimulating activity of the bath fluid was due to prostaglandin-like material, pooled bath fluid of mucosa-scraped preparations was assayed for its prostaglandin content before and after extraction plus chromatography. All (96.4 ± 4.6%; mean ± s.d.) of the rat stomach stimulating activity was accounted for by prostaglandin-like material.

Influence of contraction or relaxation of the jejunum on the output of prostaglandin-like material

Contraction of the tissue induced by acetylcholine (0.2–1 µg/ml) for 30 min (6 experiments) did not consistently increase the prostaglandin production by intact preparations of the rabbit jejunum. Physostigmine (0.1–1 µg/ml; 6 experiments) induced a strong contraction of the tissue; often the bath fluid became clouded with tissue debris. In 2 experiments in which the lumen of the jejunum was open, physostigmine caused an increase in output of prostaglandin-like material (2–5 times). However,

Table 1 Total (mean ± s.e. mean) output of prostaglandin-like material (expressed as ng prostaglandin E_2 equivalents/g wet weight for the specified min collection period) from different preparations of the rabbit jejunum

		Prostaglandin output (ng/g wet weight tissue)			
Preparation (number of experiments)	*Mean weight (g)*	*1st* (0–30 min)	*3rd* (60–90 min)	*6th* (150–180 min)	*9th* (240–270 min)
Intact jejunum (11)	1.7 ± 0.1	11.8 ± 1.6	7.8 ± 1.5	16.8 ± 5.0	21.5 ± 7.0
Mucosa scraped (8)	0.9 ± 0.09	259.0 ± 69.7	113.8 ± 20.4	102.0 ± 8.9	158.6 ± 31.5
Longitudinal muscle strip (6)	0.12 ± 0.02	628.2 ± 214.7	238.2 ± 84.9	63.1 ± 25.8	27.3 ± 11.1
Intact jejunum stored for 48 h at 4°C (5)	1.7 ± 0.2	65.2 ± 10.5	68.2 ± 25.1	85.2 ± 32.1	109.8 ± 32.7
Intact jejunum stored for 48 h with indomethacin (10 µg/ml) (5)	1.5 ± 0.04	9.6 ± 4.0	20.7 ± 9.8	68.5 ± 30.9	101.3 ± 44.3

For statistical analysis, see text.

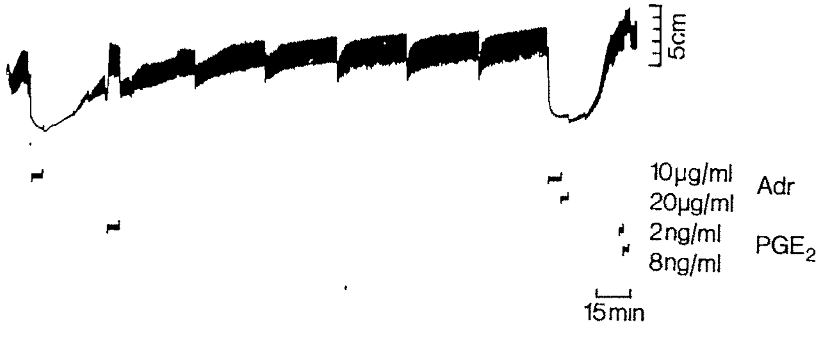


Figure 1 Rise in tone and effect of changing the bath fluid during the incubation of the rabbit jejunum in an isolated organ bath The degree of tone at different times of the experiment was estimated by administration of adrenaline (Adr). Prostaglandin E_2 (PGE$_2$) contracted the tissue and the response diminished as the tone of the tissue increased. Black dots indicate change of bath fluid. Time 15 min; vertical scale 5 cm (which represents a muscle shortening of 1.25 cm).

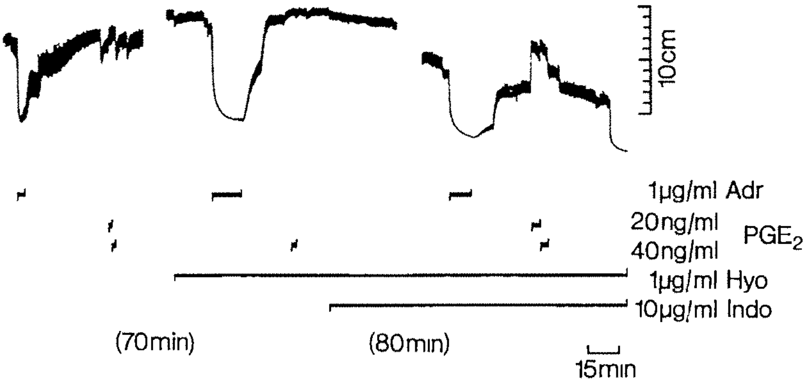


Figure 2 Effect of hyoscine (Hyo; 1 µg/ml) and indomethacin (Indo; 10 µg/ml) on the tone of the rabbit jejunum (mucosa scraped) suspended in an organ bath. The amount of tone at different times of the experiment was estimated by administration of adrenaline (Adr, 1 µg/ml) The tone of the tissue was already high in the beginning of the experiment and prostaglandin E_2 (PGE$_2$) produced a contraction only at doses of 20 and 40 ng/ml Hyoscine produced a small relaxation but did not prevent the tissue from gaining further tone. Indomethacin caused a gradual fall in tone, after which the responses to prostaglandin E_2 were enhanced. The figures between brackets represent the time elapsed between different parts of the tracing Black dots indicate change of the bath fluid. Time 15 min; vertical scale 10 cm (which represents a muscle shortening of 2.5 cm).

when the lumen of the tissue was closed to avoid expulsion of luminal contents into the bath fluid, the bath fluid from physostigmine-treated and control preparations contained similar quantities of prostaglandin-like material (2 experiments). Similarly, no increased prostaglandin output was seen in this system when physostigmine was administered after the tissue had been contracted several times by acetylcholine (1 µg/ml). No increase in the outut of prostaglandin-like material was observed during contractions of strips of longitudinal smooth muscle induced by acetylcholine (0.1–0.2 µg/ml; 2 experiments) or physostigmine (1 µg/ml; 2 experiments).

Arachidonic acid (10 µg/ml; 9 experiments) present in the organ bath for 30 min caused contraction of intact as well as of mucosa-scraped preparations. The contraction, which was quite instantaneous and which equalled a contraction induced by 15–20 ng/ml prostaglandin E_2, waned over 30 min in 6 out of 9 experiments, but was sustained in the remaining 3 experiments. There was no increase in output of prostaglandin-like material when arachidonic acid was

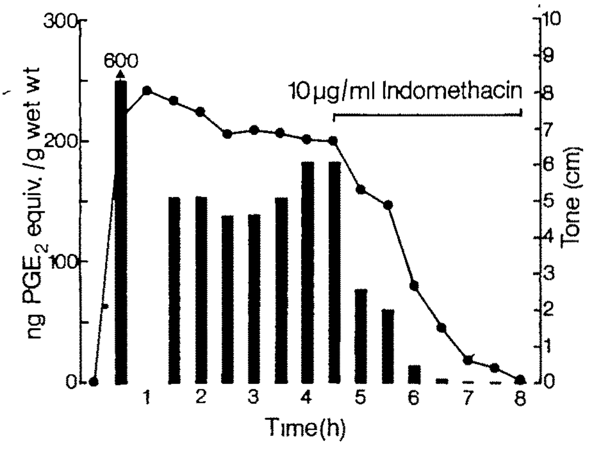


Figure 3 Effect of indomethacin (10 µg/ml) on the tone of, and production of prostaglandin-like material by, the rabbit isolated jejunum (scraped mucosa) suspended in an organ bath. The variation of the tone above the level seen after mounting the tissue in the bath was measured in cm displacement of the pen on the recorder (10 cm displacement corresponds to a muscle shortening of 2.5 cm) and is represented by the line. The total output of prostaglandin-like material during the 30 min collection periods was measured in ng prostaglandin E_2 (PGE$_2$) equivalents per g wet weight and is represented by the columns. Indomethacin reduced both the production of prostaglandin-like material and the tone of the tissue. After 4 h of treatment, the prostaglandin production had fallen to undetectable levels and the tone was reduced to the same level as that seen immediately after suspending the tissue in the bath. The time after suspending the tissue in the organ bath is indicated in hours.

added either at the beginning of the experiment or after the tissue had been in the organ bath for a few hours. Prostaglandin E_2 (10 ng/ml; 6 experiments) caused a sustained contraction of the preparation and after 30 min, all (105 ± 14.2; mean ± s.d.) of the added activity could be recovered from the bath fluid.

Adrenaline (2 µg/ml; 2 experiments) or noradrenaline (2 µg/ml; 2 experiments) strongly relaxed the jejunum, whereas hyoscine (0.1 µg/ml; 2 experiments) produced only a slight reduction of the tone. No consistent change in the output of prostaglandin-like material occurred during the administration of these drugs.

Relationship between tone of the rabbit isolated jejunum and prostaglandin production

The tone of the rabbit jejunum preparation tended to increase throughout the experiment, reaching a plateau after 3–5 hours. This increase in tone was partially reversed by each replacement of bath fluid (Figure 1). During the 30 min collection period the tone then gradually recovered reaching a slightly higher level than previously until a plateau was reached. The addition of a mixture of methysergide, mepyramine and hyoscine (0.1–0.2 µg/ml; 6 experiments) or hyoscine alone (0.1–1 µg/ml; 3 experiments) to the bath fluid did not prevent the tissue from gaining tone and, as shown in Figure 2, hyoscine (0.1–1 µg/ml; 7 experiments) only temporarily decreased the tone already developed.

As the tone of the tissue rose, so there was a gradual decrease in the amplitude of contractions produced by prostaglandin E_2 (Figures 1 and 2) or acetylcholine: the concentration of the agonists had therefore to be increased four to tenfold to obtain a contraction comparable with the one produced at the start of the experiment. In experiments in which the jejunum was superfused, there was also a gradual increase in tone over a similar time period.

Indomethacin (10 µg/ml) induced a gradual loss in tone (Figure 2) and a diminution in output of prostaglandin-like material. The close relationship between tone and prostaglandin production is shown for a single tissue in Figure 3. After indomethacin treatment adrenaline produced a much smaller relaxation, indicating a small degree of residual tone. This could be further reduced by addition of hyoscine but was insensitive to the administration of methysergide or mepyramine.

The longitudinal smooth muscle strip relaxed completely with indomethacin (1 µg/ml) within 30 min and no further relaxation could be obtained with adrenaline (1–10 µg/ml).

In preparations in which the tone had been substantially reduced by indomethacin, it could be restored by the addition to the bath of a prostaglandin E_2 (Figure 2). Furthermore, after indomethacin treatment, the contractions induced by prostaglandin E_2 or acetylcholine were somewhat enhanced as compared with the contractions obtained before indomethacin. This enhancement was most pronounced in the longitudinal muscle strip and in the jejunum with scraped mucosa (Figure 2). In some tissues, the contraction by arachidonic acid was blocked after 30 min of indomethacin administration, in others not until after 1 to 2 hours.

Bubbling the bath with N_2 rather than O_2 (10 experiments) was accompanied by a relaxation of the tissue and a 50–75% reduction in the production of prostaglandins (3 experiments; Figure 4). Bubbling once more with O_2 restored the prostaglandin production and was accompanied by a contraction of the tissue. Contractions induced by acetylcholine or prostaglandin E_2 were not sustained during N_2 and faded within 3 min despite the continuous presence of the drug.

Addition of 2,4-dinitrophenol (20 µg/ml:

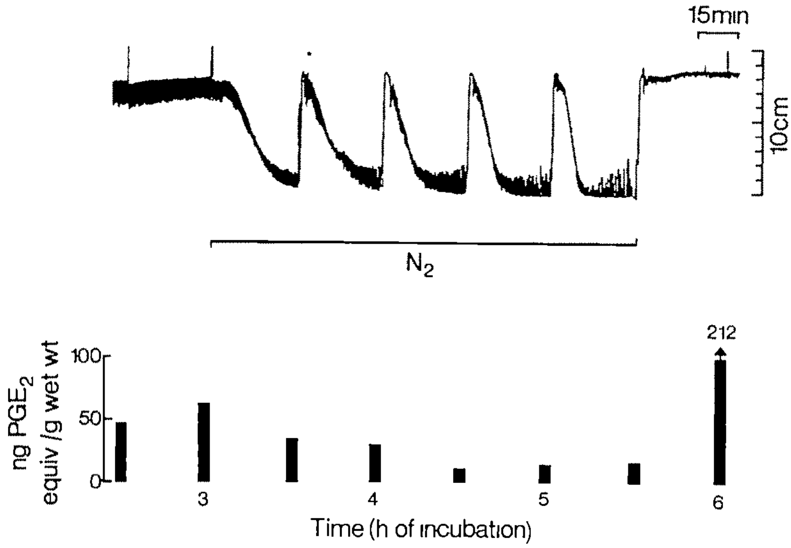


Figure 4 Effect of N_2 on the tone (upper trace) of the rabbit jejunum (mucosa scraped) suspended in an organ bath. Production of prostaglandin is shown in the lower part of the figure, expressed as ng prostaglandin E_2 (PGE_2) equivalents per gram wet weight of tissue for 30 min collection periods. The Krebs solution was bubbled with N_2 after the tissue had been incubated for 3 hours. Concomitantly with the fall in tone the output of prostaglandin-like material into the bath fluid decreased Bubbling once more with O_2 restored the tone and the production of prostaglandin-like material. Black dots indicate the change of bath fluid (which caused a temporary muscle contraction). Time scale 15 min, the time (hours of incubation) indicates the time after suspending the tissue in the organ bath. Vertical scale 10 cm which represents a muscle shortening of 2.5 cm.

3 experiments) or changing the bath fluid to calcium-free Krebs solution (2 experiments) also relaxed the tissue. The output of prostaglandins was not affected by these treatments. After 2,4-dinitrophenol, prostaglandin E_2 no longer caused a contraction, whereas acetylcholine caused a quick non-sustained contraction which was also reduced in amplitude. After removal of the 2,4-dinitrophenol, there was an immediate restoration of the tone and a reversal of the effects on contractions induced by prostaglandin or acetylcholine.

Discussion

As found for other isolated intestinal muscle, pieces of rabbit isolated jejunum release prostaglandins into the Krebs solution in which they are bathed. All of our experiments are consistent with the concept that prostaglandin generation and consequent release is proportional to the amount of manipulation of or damage to the tissue. For example, the sample of bath fluid collected during the first half-hour contained higher amounts of prostaglandins, especially when a strip of longitudinal muscle was used. Others have noted that manipulation of tissues, such as handling of the dog spleen *in vivo* (Ferreira & Vane, 1967) or stroking the external surface of the guinea-pig lungs *in vitro* (Piper & Vane, 1971) release prostaglandins or prostaglandin-like substances. The prostaglandin output from pieces of rabbit jejunum also increased as the experiment progressed and this again could be an expression of a gradual deterioration of the integrity of the preparation: loss of structural integrity was already observable 15 min after isolation of the rat intestine in an organ bath (Levine, McNary, Kornguth & LeBlanc, 1970). Increased release of prostaglandins with time has also been observed during incubation of the bovine isolated iris (Posner, 1970) and during perfusion of the cat isolated spleen (Ferreira, Moncada & Vane, 1973) and the dog kidney (Itskovitz, Stemper, Pacholczyk & McGiff, 1973). Certainly, in our hands any procedure which damaged the tissues was followed by an increase in prostaglandin output. The increase was seen both from damaged muscle and damaged mucosa; it was interesting in this respect that the strips of longitudinal muscle released declining amounts of prostaglandins. This may have been because, after the initial trauma of dissection, the tissue did not suffer progressive damage through lack of nutrients. However, it is also possible that the bulk of prostaglandins is derived from the mucosal layer:

four times more prostaglandins can be extracted from the mucosa of the human stomach than from the submucosa, whereas only traces of prostaglandin activity could be found in the muscle layer (Bennett, Murray & Wyllie, 1968).

Contraction or relaxation of the rabbit jejunum did not affect prostaglandin output. Thus, any prostaglandin production associated with increased or decreased muscle activity is small compared with the continuous output measured here. The increase in output seen with physostigmine was accompanied by and perhaps explained by the expulsion of tissue debris from the lumen of the gut. There is now substantial evidence that contractions of isolated smooth muscle induced by arachidonic acid are due to prostaglandins generated within the tissue (see Ferreira & Vane, 1974). In these experiments also, addition of arachidonic acid to the bath fluid caused contraction of the tissue, but did not lead to an increase in prostaglandin production. A similar observation was made by Posner (1971) in bovine iris. Adequate amounts of substrate must already be present and the increased production which leads to further contraction must be small compared with the continuous output. The increase in prostaglandin output observed during the course of the experiment is unlikely to be the result of an increased liberation of precursors by phospholipase activity, as proposed by Bartels, Kunze, Vogt and Wille (1970) working with perfused frog intestine.

Since output of prostaglandins from a tissue probably represents a balance between production and metabolism within the tissue, an increased output could also be due to a decreased inactivation. In this context, Blackwell, Flower & Vane (1975) have shown that prostaglandin 15-OH dehydrogenase is a short-lived enzyme, so that continued activity depends upon continued enzyme production. Thus, isolated tissues may well lose dehydrogenase activity within an hour or so. However, addition of prostaglandin E_2 to the bath fluid showed that even from the very beginning of the experiment, there was little or no measurable metabolism by the rabbit isolated jejunum of the exogenously added prostaglandins. Thus, the gradual increase in the prostaglandin content of the bath fluid after 3 h is probably due to an increased synthesis rather than a gradual decline in the metabolism of the prostaglandins.

The increased production may be due to an increase in enzyme level or enzyme availability or to an increase in the availability of O_2 to the synthesizing system. Molecular oxygen is certainly necessary for prostaglandin production (Samuelsson, Granström & Hamberg, 1967; Nugteren, Beerthuis & Van Dorp, 1967) and we have shown that bubbling the organ bath with N_2 reduced prostaglandin production by the rabbit jejunum. At the same time, the tone of the tissue fell, probably because O_2 is not only required for the production of prostaglandins but also for their activity (Coceani & Wolfe, 1966; Eckenfels & Vane, 1972).

The involvement of prostaglandin production in the maintenance of the tone of this isolated tissue was further shown by the actions of indomethacin, which reduced not only the prostaglandin output but also the tone of the preparation. These effects of indomethacin are probably due to specific inhibition of prostaglandin biosynthesis, for much higher concentrations are needed to inhibit agonist-induced smooth muscle contractions (Northover, 1971; Sorrentino, Capasso & Di Rosa, 1972) or to uncouple oxidative phosphorylation (Whitehouse, 1964). Dinitrophenol, a much more powerful uncoupler of oxidative phosphorylation, also relaxes smooth muscle but we have shown that output of prostaglandins is not affected. However, dinitrophenol inhibits the response of the tissue to prostaglandins and this action could explain its effect on the inherent tone of the preparation, rather than an action on the energy supply to the tissue (Born & Bülbring, 1955).

Our experiments with hyoscine and indomethacin show that the tone of the rabbit isolated jejunum is maintained by at least three different mechanisms: one mechanism, which accounts for about 5–10% of the tone, is hyoscine-sensitive, and is presumably due to the continuous generation of acetylcholine; a second one, which accounts for about 70–80% of the tone, is indomethacin-sensitive and therefore related to the production of prostaglandins. The remainder of the tone after treatment of the tissue with hyoscine and indomethacin is insensitive to methysergide and mepyramine, but could be abolished by bubbling the organ bath with N_2.

The relationship between tone and prostaglandin production is further supported by work with indomethacin on other isolated smooth muscle preparations (Farmer, Farrar & Wilson, 1972; Botting & Salzmann, 1974), with other prostaglandin synthetase inhibitors (Eckenfels & Vane, 1972; Davison, Ramwell & Willis, 1972) and with prostaglandin antagonists (Bennett & Posner, 1971; Ganesan & Karim, 1973).

Our results do not preclude the possibility that prostaglandin production in the rabbit jejunum *in vivo* also contributes to muscle tone and movement. However, the fact that manipulation and trauma increase prostaglandin production in the isolated preparation suggests that the prostaglandin production which we measure and which maintains the tone of the isolated preparation could be linked with removal of the tissue from the body and its incubation in an artificial medium. Such a conclusion stresses the importance of prostaglandins in pathophysiological conditions; indeed the involvement of prostaglandins in the inflammatory process (Willis, 1969) is another indication of involvement of prostaglandins in pathological conditions. Thus prostaglandin production by intestine may be induced, not only by incubation in an isolated organ bath, but also by inflammation of the intestine *in vivo* as seen in some intestinal infections, mechanical obstructions, ischaemic necrosis, irradiation, etc. All of these conditions may lead to increased prostaglandin production which would increase motility of the gut and could also cause diarrhoea.

We thank Miss V. Pontieri and Mr M.J. Parnham for technical assistance; Dr J. Pike of Upjohn, Kalamazoo, U S.A. for the gift of prostaglandins; Merck, Sharp & Dohme for the gift of indomethacin and The Wellcome Trust for a grant supporting this work.

References

AMBACHE, N. (1954). Separation of the longitudinal muscle of the rabbit's ileum as a broad sheet. *J. Physiol., Lond.,* **125**, 53P

BARTELS, J., KUNZE, H., VOGT, W. & WILLE, G. (1970). Prostaglandin: liberation from and formation in perfused frog intestine. *Naunyn Schmiedeberg's Arch. Pharmak.,* **266**, 199–207.

BENNETT, A, FRIEDMANN, C.A. & VANE, J.R (1967). Release of prostaglandin E_1 from the rat stomach. *Nature, Lond.,* **216**, 873–876.

BENNETT, A., MURRAY, J G. & WYLLIE, J.H. (1968). Occurrence of prostaglandin E_2 in the human stomach, and a study of its effects on human isolated gastric muscle. *Br. J. Pharmac. Chemother.,* **32**, 339–349.

BENNETT, A. & POSNER, J. (1971). Studies on prostaglandin antagonists. *Br. J. Pharmac.,* **42**, 584–594.

BLACKWELL, G.J., FLOWER, R.J. & VANE, J.R. (1975). Rapid reduction of prostaglandin 15-hydroxy-dehydrogenase (PGDH) activity in rat tissues after treatment with protein synthesis inhibitors. *Br. J. Pharmac.,* **55**, 233–238.

BORN, G V.R & BÜLBRING, E (1955). The effects of 2:4 dinitrophenol (DNP) on the smooth muscle of guinea-pig's taenia coli. *J. Physiol., Lond.,* **127**, 626–635.

BOTTING, J H. & SALZMANN, R (1974). The effect of indomethacin on the release of prostaglandin E_2 and acetylcholine from guinea-pig isolated ileum at rest and during field stimulation. *Br. J. Pharmac.,* **50**, 119–124.

COCEANI, F. & WOLFE, L.S. (1966). On the action of prostaglandin E_1 and prostaglandins from brain on the isolated rat stomach. *Can. J. Physiol. Pharmac.,* **44**. 933–950.

COCEANI, F., PACE-ASCIAK, C., VOLTA, F & WOLFE, L.S. (1967). Effect of nerve stimulation on prostaglandin formation and release from the rat stomach. *Am. J. Physiol.,* **213**, 1056–1064.

DAVISON, P., RAMWELL, P.W. & WILLIS, A.L. (1972). Inhibition of intestinal tone and prostaglandin synthesis by 5, 8, 11, 14-tetraynoic acid. *Br. J. Pharmac.,* **46**, 547–548P.

ECKENFELS, A. & VANE, J.R. (1972). Prostaglandins, oxygen tension and smooth muscle tone. *Br. J. Pharmac.,* **45**, 451–462.

FARMER, J.B., FARRAR, D.G. & WILSON, J. (1972). The effect of indomethacin on the tracheal smooth muscle of the guinea-pig. *Br. J. Pharmac.,* **46**, 536–537P.

FERREIRA, S.H., HERMAN, A. & VANE, J.R. (1972). Prostaglandin generation maintains the smooth muscle tone of the rabbit isolated jejunum. *Br. J. Pharmac.,* **44**, 328–330P.

FERREIRA, S.H., MONCADA, S. & VANE, J.R. (1973). Some effects of inhibiting endogenous prostaglandin formation on the responses of the cat spleen. *Br. J. Pharmac.,* **47**, 48–58.

FERREIRA, S.H. & VANE, J.R (1967). Prostaglandins: their disappearance from and release into the circulation. *Nature, Lond.,* **216**, 868–873.

FERREIRA, S.H. & VANE, J.R. (1974). Aspirin and prostaglandins. In *The Prostaglandins,* ed. Ramwell, P.W., pp. 1–47. New York: Plenum Press.

GADDUM, J.H (1953). The technique of superfusion. *Br. J. Pharmac. Chemother.,* **8**, 321–326.

GANESAN, P.A. & KARIM, S.M.M (1973). Polyphloretin phosphate temporarily potentiates prostaglandin E_2 on the rat fundus, probably by inhibiting PG 15-hydroxy-dehydrogenase. *J. Pharm. Pharmac.,* **25**, 229–233.

GILMORE, N., VANE, J.R. & WYLLIE, J.H. (1968). Prostaglandins released by the spleen. *Nature, Lond.,* **218**, 1135–1140.

GRÉEN, K. & SAMUELSSON, B. (1964). Thin-layer chromatography of the prostaglandins. *J. Lipid Res.,* **5**, 117–120.

ITSKOVITZ, H.D., STEMPER, J., PACHOLCZYK, D. & McGIFF, J.C. (1973). Renal prostaglandins: determinants of intrarenal distribution of blood flow. *Clin. Sci. Mol. Med.,* **45** (Suppl. 1), 321–324.

LEVINE, R.R., McNARY, W.F, KORNGUTH, P.J. & LeBLANC, R. (1970). Histological re-evaluation of everted gut technique for studying intestinal absorption. *Europ. J. Pharmac.,* **9**, 211–219.

NORTHOVER, B.J. (1971). Mechanism of the inhibitory action of indomethacin on smooth muscle. *Br. J. Pharmac.,* **41**, 540–551.

NUGTEREN, D.H., BEERTHUIS, R.K. & VAN DORP, D.A. (1967). Biosynthesis of prostaglandins. In *Proc. II Nobel Symp.,* ed. Bergström, S. & Samuelsson, B., pp. 45–50. Stockholm: Interscience Publishers.

PATON, W.D.M. (1957). A pendulum auxotonic lever. *J. Physiol., London,* **137**, 35–36P.

PIPER, P.J. & VANE, J.R. (1971). The release of prostaglandins from lung and other tissues. *Ann. N.Y. Acad. Sci.,* **180**, 363–383.

POSNER, J. (1970). The release of prostaglandin E_2 from the bovine iris. *Br. J. Pharmac.,* **40**, 163–164P.

POSNER, J. (1971). The actions of drugs and nerve stimulation on isolated intraocular smooth muscles. Ph.D. Thesis, Univ. London.

SAMUELSSON, B., GRANSTRÖM, E. & HAMBERG, M. (1967). On the mechanism of biosynthesis of prostaglandins. In *Proc. II Nobel Symp.*, ed. Bergström, S. & Samuelsson, B., pp. 31–34. Stockholm: Interscience Publishers.

SORRENTINO, L., CAPASSO, F. & DI ROSA, M. (1972). Indomethacin and prostaglandins. *Europ. J. Pharmac.*, **17**, 306–308.

SUZUKI, T. & VOGT, W. (1965). Prostaglandine in einem Darmbewegungen und des Darmvagusreizes. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **252**, 68–78.

VANE, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs. *Nature, New Biol.*, **231**, 232–235.

VANE, J.R. & WILLIAMS, K.I. (1973). The contribution of prostaglandin production to contractions of the isolated uterus of the rat. *Br. J. Pharmac.*, **48**, 629–639.

VOGT, W. (1949). Über die stoffliche Grundlage der Darmbewegungen und des Darmvagusreizes. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.*, **208**, 221–223.

WILLIS, A.L. (1969). Parallel assay of prostaglandin-like activity in rat inflammatory exudate by means of cascade superfusion. *J. Pharm. Pharmac.*, **21**, 126–128.

WEILAND, W. (1912). Zur Kenntnis der Entstehung der Darmbewegung. *Pflügers Arch. ges. Physiol.*, **147**, 171–196.

WHITEHOUSE, M.W. (1964). Uncoupling of oxidative phosphorylation by some arylacetic acids (anti-inflammatory or hypocholesterolemic drugs). *Nature, Lond.*, **201**, 629–630.

(Received October 16, 1975.
Revised November 14, 1975.)

Br. J. Pharmac. (1976), **56**, 479–484

THE EFFECTS OF DIMERCAPTOSUCCINIC ACID ON THE EXCRETION AND DISTRIBUTION OF MERCURY IN RATS AND MICE TREATED WITH MERCURIC CHLORIDE AND METHYLMERCURY CHLORIDE

L. MAGOS
MRC Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey

1 All five rats in a group survived if dimercaptosuccinic acid (DMSA), a water soluble derivative of 2,3-dimercaptopropanol (BAL), was given in doses of 10–40 mg/kg intraperitoneally 30 min, 4 and 24 h after administration of 2.4 mg/kg Hg as $HgCl_2$, whereas three out of a group of five died if DMSA was not given. DMSA 20 mg/kg increased urinary excretion and decreased the body burden significantly more than 10 mg/kg DMSA, but further doubling of the dose had only marginal effects.

2 DMSA was able to reduce body burden and increase urinary excretion of Hg when intraperitoneal treatment started eight days after the subcutaneous administration of $HgCl_2$.

3 DMSA was effective in decreasing body burden and the brain concentration of Hg in rats dosed orally with methylmercury (MeHgCl) when intraperitoneal treatment started with 40 mg/kg DMSA 24 h after Hg. Increase in the urinary excretion of mercury was responsible for the decrease in body burden.

4 DMSA was effective when given in the drinking water of rats or mice both against inorganic Hg and MeHgCl. In mice treated intraperitoneally with MeHgCl, DMSA 19.5 µg/ml in the drinking water caused a significant decrease in the body burden and increase in the excretion of Hg.

5 DMSA was about four times more efficient than D-penicillamine in decreasing the body burden of Hg. As their toxicity is in the same range, the higher efficiency of DMSA offers a larger margin of safety for the mobilization of Hg.

Introduction

After the discovery that 2,3-dimercaptopropanol (BAL) is effective against $HgCl_2$ intoxication both in experimental animals (Braun, Lusky & Calvery, 1946) and in man (Longcope & Leutscher, 1949), the search has never stopped for a more efficient and less toxic chelating agent. This effort led to the discovery that D-penicillamine (Aposhian, 1958) and N-acetyl-DL-penicillamine (Aposhian & Aposhian, 1959) were both effective antidotes against $HgCl_2$ in animal experiments, though their efficiency in man for the treatment of $HgCl_2$ intoxication is not consistent (Kazantzis, Schiller, Asscher & Drew, 1962; Teisinger & Srbova, 1964; Javett & Kaplan, 1968; Kark, Poskanzer, Bullock & Boylen, 1971).

The modification of BAL led to the synthesis of 2,3,-dimercaptopropanol-sulphonate which under the name of Unithiol is used in the USSR and has proved to be an effective antidote against $HgCl_2$ (Belonozhko, 1958). Another derivative of BAL: meso-dimercaptosuccinic acid (DMSA) (HOOC-CHSH-CHSH-COOH) was used recently for the mobilization of Hg in mice and guinea-pigs both after the administration of $HgCl_2$ and MeHgBr (Friedheim & Corvi, 1975). After a single dose of $HgCl_2$ or MeHgBr two daily doses of 100 mg/kg DMSA given intraperitoneally decreased the kidney, liver and the brain content of mercury in mice. In guinea-pigs the same effect was tested and confirmed with 250 to 1000 mg/kg DMSA. Equal doses of D-penicillamine were less effective, though the LD_{50} for D-penicillamine (Friedrich & Zimmermann, 1975) and for DMSA (Friedheim & Corvi, 1975) is the same: between 3.0 and 4.0 g/kg. The present study is an extension of Friedheim & Corvi's work. Significantly smaller doses of DMSA were used and their effect on the survival of rats after the administration of $HgCl_2$ and on the body burden and the excretion of Hg was followed in rats and mice, both after the administration of $HgCl_2$ and MeHgCl. Because of the convenience of oral administration, the effect of DMSA was also tested when it was administered in the drinking water.

Methods

White male rats (Porton-Wistar strain) of approximately 200 g body weight and white male mice

(LACA strain) of approximately 20 g body weight were used.

Hg was injected either as $HgCl_2$ (Analar, B.D.H. Laboratory Chemicals, Poole) or MeHgCl (K & K Laboratories, Plainview, N.Y.) labelled with $^{203}HgCl$ or $Me^{203}HgCl$ (Radiochemical Centre, Amersham). $Me^{203}HgCl$ supplied by the Radiochemical Centre was extracted from the aqueous solution into carbon tetrachloride which was subsequently evaporated and the residue dissolved in water. No more than 2% of the total Hg in the injection solution of MeHgCl was inorganic as estimated by the method of Magos & Clarkson (1972).

Doses expressed in Hg, volumes of the injection solutions and specific activities were as follows: in experiments with $HgCl_2$ rats were injected intraperitoneally with 2.4 mg/kg in a volume of 3.2 ml/kg (sp. act. 0.12 μCi/mg) or subcutaneously with 0.5 mg/kg in a volume of 2.0 ml/kg (sp. act. 1.0 μCi/mg); MeHgCl was given to rats by oral cannulation in a volume of 2.0 ml/kg either in a single dose of 3.36 mg/kg (sp. act. 0.26 μCi/mg) or six daily doses (with two days interval between the fourth and the fifth dose) of 6.0 mg/kg (sp. act. 0.1 μCi/mg).

Mice were given both $HgCl_2$ (sp. act. 40 μCi/mg) and MeHgCl (sp. act. 15–25 μCi/mg) in a dose of 0.5 mg/kg intraperitoneally in a volume of 10 ml saline/kg. DMSA (supplied by E.A.H. Friedheim, Geneva) was given to rats either intraperitoneally in a volume of 5 ml/kg in saline (0.9% w/v NaCl solution) or in the drinking water containing 0.5% disodium edetate (EDTA). Controls were injected with saline or given 0.5% EDTA in the drinking water. The volume of drinking water was 30 ml per rat, and almost all was consumed by the animals. Mice were given DMSA in the drinking water. Five mice drank approximately 20 ml per day from the 25 ml drinking water supplied. In some experiments mice were given D-penicillamine 2.5 mg/ml (Dista Products Ltd., Liverpool) instead of DMSA. When urine and faeces was collected two rats were placed in one metabolic cage. Faeces and urine were separated according to Östlund (1969) from five mice kept in one cage.

Details of experimental schedules are given in the Tables.

Radioactive measurements

Live animals placed in a 14 × 6 × 6.5 cm plexiglass box were counted with an efficiency of 7.7% between two NaI crystal pairs (No. N656, EKCO Electronics Ltd., Southend-on-Sea, Essex) facing each other. Faeces and urine samples, blood and liver, in beakers, were counted in the same system. Kidneys and spleen and in some experiments blood were counted in a well-shaped NaI crystal with an efficiency of 31%. Injection standards in 1 ml acid permanganate solution were counted and Hg concentrations in organs and in whole animals were calculated after corrections for geometrical differences by relating their radioactivities to standards.

Mathematical calculations

Half times were calculated from the equation

$$A = A_0 \times e^{-bt}$$

where A_0 is the injected dose, A is the body burden at time t (in days) by the use of the weighted least mean square curves of best fit essentially as described by

Table 1 The effect of dimercaptosuccinic acid (DMSA) on survival, body burden and excretion of Hg in male rats injected subcutaneously with 2.4 mg/kg Hg as $HgCl_2$

Dose of DMSA (mg/kg)	*% of initial body burden*							
	Decrease in initial body burden (mean ± s.e. mean)			*Urinary excretion*		*Faecal excretion*		*6 day mortality*
	24 h	*48 h*	*6 day*	*0–24 h*	*24–48 h*	*0–24 h*	*24–48 h*	
—	10.0 ± 1.16 (*n* = 5)	20.1 ± 3.04 (*n* = 5)	52.1; 54.9 (*n* = 2)	7.5	0.6	4.2	11.8	3/5
10	27.0 ± 2.99* (*n* = 5)	38.0 ± 2.54* (*n* = 5)	61.4 ± 2.80* (*n* = 5)	17.5	3.0	5.0	10.6	0/5
20	37.0 ± 1.61 (*n* = 5)	48.8 ± 3.99 (*n* = 5)	72.4 ± 2.34 (*n* = 5)	26.4	10.0	4.1	14.1	0/5
40	36.0 ± 2.40 (*n* = 5)	54.7 ± 3.17 (*n* = 5)	76.8 ± 1.85 (*n* = 5)	28.0	14.0	5.6	12.0	0/5

Dimercaptosuccinic acid was given intraperitoneally in saline 30 min, 4 h and 24 h after the administration of Hg in a volume of 5 ml/kg. Control animals were given the same volume of saline. When faeces and urine were collected, two animals shared one metabolic cage and excreta were collected from four animals per group.
* Significantly different ($P < 0.05$) from groups given higher doses of DMSA with one exception. at 48 h the 10 mg/kg group differs significantly only from the 40 mg/kg group.

Deming (1964) using a programmable desk type calculating machine (Hawlet-Packard 9810A calculator). Statistical differences were calculated by Student's *t* test.

Results

Table 1 shows that three of the five rats injected subcutaneously with 2.4 mg/kg Hg as $HgCl_2$ died within six days. None of the animals treated with DMSA died. Compared with the controls the body burden decreased 100% more and urinary excretion of Hg increased at least 2.5 times in the first 48 h after Hg in rats dosed with 10 mg/kg DMSA. Treatment with 20 mg/kg DMSA resulted in a further decrease in the body burden and increase in the urinary excretion of Hg. A further increase of the dose of DMSA did not affect the body burden of Hg. Faecal excretion remained unchanged by DMSA.

Table 2 shows that DMSA is able to mobilize Hg eight days after the injection of 0.5 mg/kg Hg as

Table 2 The effect of three doses of 20 mg/kg dimercaptosuccinic acid (DMSA) given intraperitoneally eight days, eight days + 6 h and nine days after the subcutaneous administration of 0.5 mg/kg Hg as $HgCl_2$ on the excretion, body burden and organ contents of Hg in male rats

	No. of rats per group	*Hg in % of the pretreatment body burden (mean ± s.e mean)* *in control*	*in DMSA treated*
Excretion			
Urine 0–24 h	10	1.8 ±0.14	9.3 ±0.44*
24–48 h	10	0.9 ±0.12	2.4 ±0.27*
Faeces 0–24 h	10	1.5 ±0.12	2.3 ±0.15*
24–48 h	10	1.1 ±0.09	1.6 ±0.06
Hg Content			
Whole body	10	92.9 ±1.38	87.0 ±1.07*
Kidneys	10	80.1 ±1.70	74.8 ±0.88*
Blood	10	0.47 ±0.03	0.45 ±0.03
Liver	10	1.5 ±0.13	1.7 ±0.10
Spleen	10	0.081 ±0.007	0.082 ±0.004

Animals were killed 48 h after the first DMSA injection. Urine and faecal samples were obtained from two animals. Calculation for blood mercury was based on the assumption that 7% of the body weight is blood.
* Significantly different from the control ($P<0.05$).

Table 3 The effect of three doses of 40 mg/kg dimercaptosuccinic acid (DMSA) given intraperitoneally on the excretion, body burden and organ contents of Hg in male rats dosed with 3.36 mg/kg Hg as MeHgCl by oral cannulation in a volume of 2 ml/kg

	Hg in % of the dose (mean ± s.e. mean) *In control*	*in DMSA-treated*
Excretion after first DMSA injection		
Urine 0–24 h	0.8	20.3
24–48 h	1.5	7.4
Faeces 0–24 h	2.9	2.1
24–48 h	3.5	2.9
Hg content		
Whole body	89.1 ±2.38	62.7 ±1.58*
Brain	0.31 ±0.011	0.22 ±0.010*
Kidneys	3.2 ±0.10	2.2 ±0.15*
Blood	41.5 ±1.00	28.6 ±1.15*
Liver	7.3 ±0.32	5.1 ±0.23*

DMSA was given 24, 30 and 48 h after mercury. Animals were killed 48 h after the first DMSA injection. There were four animals per group. Values for urine and faeces are the mean of two samples obtained from four animals.
* Significantly different ($P<0.05$) from the control.

$HgCl_2$. The effect on body burden and the urinary excretion of Hg was significant. Faecal excretion of Hg in the first 24 h also increased. At the end of the 48 h treatment period, the concentration of Hg in the blood and spleen remained the same as in the controls but DMSA significantly decreased the kidney burden of Hg.

Table 3 shows that DMSA given 24–48 h after the *per os* administration of 3.36 mg/kg Hg as MeHgCl decreased the body burden and increased the urinary excretion of Hg significantly and decreased the concentration of Hg in every organ tested including the brain.

Table 4 shows that DMSA was effective in decreasing the body burden and the concentration of Hg in brain, blood, liver and kidneys when DMSA was given in the drinking water to rats pretreated repeatedly with MeHgCl. DMSA given in the drinking water of mice treated intraperitoneally with $HgCl_2$ (Table 5) or with MeHgCl (Table 6) decreased the biological half time and increased the urinary excretion of Hg. Even 19.5 µg/ml DMSA was effective. The effect on faecal excretion was not consistent. The urinary excretion of Hg was always increased significantly by DMSA and both urinary excretion and decrease in body burden showed a dose-dependence.

D-Penicillamine 2.5 mg/ml in the drinking water of mice treated with the same dose of MeHgCl resulted in a biological half time of approximately 2.2 days. Animals excreted 16.9 ± 1.4% daily of the body burden in the urine and 4.8 ± 0.26% daily in the faeces.

Discussion

The present experiments confirmed the effectiveness of DMSA in decreasing the body burden of Hg. This effect is more pronounced after the administration of MeHgCl than after inorganic Hg. Moreover, it has been shown that DMSA exerts its protective effect not only when it was given shortly after the administration of Hg compounds but also if the treatment started after a considerable delay and by significantly lower doses than used by Friedheim & Corvi (1975). It was

Table 4 Effect of dimercaptosuccinic acid (DMSA) given in the drinking water (2.5 mg/ml) three days after the last of the six daily doses of methylmercury (MeHgCl) in male rats.

		Hg concentration in µg Hg/g (mean ± s.e. mean)					
Treatment	*No. of rats*	*Whole body 3 days after Hg*	*Whole body 9 days after Hg*	*Brain*	*Blood*	*Kidneys*	*Liver*
—	5	27.5 ± 0.69	20.1 ± 1.07	8.7 ± 0.46	99.3 ± 8.34	95.9 ± 7.34	22.5 ± 1.53
DMSA	4	26.8 ± 0.69	10.1 ± 0.15*	4.0 ± 0.17*	28.0 ± 1.22*	37.8 ± 2.3*	6.6 ± 0.69*

MeHgCl was given *per os* in a dose of 6.0 mg/kg Hg. Treatment with DMSA lasted for three days. Animals were killed nine days after the last dose of MeHgCl that is three days after the end of the DMSA treatment

* Significantly different ($P < 0.05$) from the control.

Table 5 Effect of dimercaptosuccinic acid (DMSA) on the half life and excretion of Hg in male mice

DMSA in the drinking water (mg/ml)	*Half time (days)*		*Daily excretion in % of body burden (mean ± s.e. mean)*	
	Mean	*Limits of s.e. mean*	*In urine*	*in faeces*
—	3.51 *n* = 5	3.27–3.79	7.52 ± 1.85 *n* = 4	8.67 ± 0.80 *n* = 4
0.156	1.99*† *n* = 5	1.94–2.04	11.08 ± 1.17 *n* = 4	14.77 ± 1.03* *n* = 4
0.625	1.53* *n* = 5	1.50–1.56	18.00 ± 2.87* *n* = 4	14.65 ± 0.88* *n* = 4

$HgCl_2$ was given intraperitoneally in a dose of 0.5 mg Hg/kg, DMSA was given in the drinking water. Body burden was counted for five days and excreta collected for four days after treatment with Hg. Urine or faecal samples were collected from five mice.

* Significantly different ($P < 0.05$) from the control; † significantly different from the higher dose groups.

effective not only after parenteral administration but also if it was given in the drinking water. In the drinking water of mice a concentration as low as 19.5 μg/ml which corresponds to a dose of 4.0 mg/kg had a significant effect on the half time of the body burden after the administration of MeHgCl. D-Penicillamine given in similar experimental circumstances was at least four times less effective than DMSA (Magos & Stoytchev, 1969).

The decrease in body burden paralleled the decrease in the mercury concentration in the critical organs, in the kidneys, and in the brain in the case of MeHgCl. As Table 2 shows, the amount of Hg removed from the kidneys of inorganic Hg-treated rats was completely excreted and not redistributed in the body. In the MeHgCl experiments the kidney in relation to body burden contained 25 times less Hg; the loss of Hg was not restricted to one organ but to every tissue tested.

DMSA both in sublimate- and MeHgCl-treated rats decreased the body burden mainly by increasing the urinary excretion of Hg. Faecal excretion of Hg was either increased or not affected by DMSA. Swensson & Ulfvarson (1967) observed the same effect with BAL. However, when data in Table 2 are compared with experiments carried out in similar circumstances (Magos & Stoytchev, 1969) it is clear that, on a molar basis DMSA was slightly more efficient in increasing the urinary excretion of Hg than BAL and at least four times as efficient as D-penicillamine but unlike BAL and D-penicillamine, DMSA did not increase the blood concentration of Hg. A redistribution caused by BAL leading to higher Hg concentration in the brain in methylmercury-treated rats (Berlin, Jerksell & Nordberg, 1965) is the major contraindication in the use of BAL to decrease the body burden of methylmercury. Another point which needs consideration is that the toxicity of BAL is high, the LD_{50} for

Table 6 Effect of dimercaptosuccinic acid (DMSA) on the half life and excretion of methylmercury (MeHgCl) in male mice

	Treatment with methylmercury			
DMSA in drinking water (mg/ml)	*Half time (days)*		*Daily excretion in % of the body burden (mean ± s.e. mean)*	
	Mean	*Limits of s.e. mean*	*in urine*	*in faeces*
—	10.88 *n*=20	10.47 – 11.33	1.94±0.19 *n*=16	3.89±0.24 *n*=6
0.0120	6.04*† *n*=5	5.77 – 6.35	2.95±0.30*† *n*=4	5.50±0.52* *n*=4
0.039	4.29*† *n*=5	4.08 – 4.52	9.28±0.69* *n*=4	4.37±0.38 *n*=4
0.078	2.64*† *n*=5	2.57 – 2.72	10.63±0.97*† *n*=4	7.10±1.20* *n*=4
0.1562	2.42*† *n*=5	2.40 – 2.43	14.40±1.27* *n*=4	4.25±0.15 *n*=4
0.313	1.84*† *n*=10	1.75 – 1.94	15.30±1.18*† *n*=8	4.34±0.49 *n*=8
0.625	1.21*† *n*=10	1.15 – 1.28	23.50±1.92* *n*=8	5.21±0.70 *n*=8
1.25	0.784* *n*=9	0.782 – 0.786	23.27±1.89* *n*=4	7.25±1.40* *n*=4
2.50	0.735* *n*=5	0.690 – 0.786	27.23±1.81*† *n*=4	5.53±0.94 *n*=4
5.00	0.638* *n*=5	0.613 – 0.665	43.03±3.39* *n*=4	5.37±1.579 *n*=4

MeHgCl was given intraperitoneally in a dose of 0.5 mg Hg/kg, DMSA was given in the drinking water. Body burden was counted and excreta collected for four days after treatment with methylmercury. Every urine and faeces sample was collected from five mice.

* Significantly different ($P < 0.05$) from the control; † significantly different from the next higher dose; for faecal excretion only significant difference is given between control and treated groups.

mice being 100 mg/kg (Durlacher, Bunting, Harrison, Ordway & Albrink, 1946).

Summarizing the advantage of DMSA, the following points seem particularly significant. Firstly DMSA, like D-penicillamine and Unithiol, is a water-soluble compound which makes administration convenient. Secondly its toxicity is in the same range as that of D-penicillamine and it is approximately 35 times less toxic than BAL. The LD_{50} for Unithiol in mice in 1270 mg/kg (Kostygov, 1958) which makes Unithiol approximately three times more toxic than DMSA. Thirdly, compared with D-penicillamine the efficiency of DMSA on the basis of mg/kg is four times higher in decreasing the body burden and increasing the urinary excretion of Hg. Consequently in clinical trials lower doses of DMSA may be used than the usual dose of D-penicillamine.

The author would like to express his thanks to Dr E.A.H. Freidheim, Geneva, for the generous supply of dimercaptosuccinic acid and A.R. Hudson for technical assistance.

References

APOSHIAN, H.V. (1958). Protection by D-penicillamine against the lethal effects of mercury chloride. *Science*, **128**, 93.

APOSHIAN, H V. & APOSHIAN, M M. (1959). N-acetyl-DL-penicillamine, a new oral protective agent against the lethal effects of mercuric chloride. *J. Pharmac. exp. Ther.*, **126**, 131–135.

BELONOZHKO, G A. (1958). Therapeutic action of Unithiol in poisoning with inorganic mercury compounds. *Farmakol. i Toxikol.*, **21**, No. 3, 69–73.

BERLIN, M., JERKSELL, L.-G. & NORDBERG, G. (1965). Accelerated uptake of mercury by brain caused by 2,3-dimercaptopropanol (BAL) after injection into the mouse of a methylmercury compound. *Acta pharmac. Tox.*, **23**, 312–320.

BRAUN, H A., LUSKY, L.M. & CALVERY, H.O. (1946). The efficacy of 2,3-dimercaptopropanol (BAL) in the therapy of poisoning by compounds of antimony, bismuth, chromium, mercury and nickel. *J. Pharmac. exp. Ther.*, **87**, suppl. 119–125.

DEMING, W.E. (1964). *Statistical Adjustment of Data*. New York: Dover Publications.

DURLACHER, S H., BUNTING, H., HARRISON, H.E. ORDWAY, N K. & ALBRINK, W S. (1946). The toxicological action of 2,3-dimercaptopropanol (BAL). *J. Pharmac. exp. Ther.*, **87**, suppl. 28–32.

FRIEDHEIM, E. & CORVI, C (1975). Meso-dimercaptosuccinic acid, a chelating agent for the treatment of mercury poisoning. *J. Pharm. Pharmac.*, **37**, 624–626.

FRIEDRICH, L. & ZIMMERMANN, F. (1975). Zur Pharmakologie von Penicillamin. *Arzneimittel-Forsch.*, **25**, 172–168.

JAVETT, S.N. & KAPLAN, G. (1968). Acrodynia treated with D-penicillamine. *Amer. J. Dis. Child.*, **115**, 71–73.

KARK, R.A, POSKANZER, D.C., BULLOCK, J.D & BOYLEN, G (1971). Mercury poisoning and its treatment with N-acetyl-α-penicillamine. *New Engl. J. Med.*, **285**, 10–16.

KAZANTZIS, G., SCHILLER, K F.R., ASSCHER, A.W. & DREW, R.G (1962). Albuminuria and the nephrotic syndrome following exposure to mercury and its compounds. *Quart. J. Med.*, **28**, 403–418.

KOSTYGOV, N.M. (1958). The antidotal action of mercaptosuccinic acid and Unithiol against mercury. *Farmakol. i Toxickol.*, **21**, No. 3, 64–69.

LONGCOPE, W.T. & LEUTSCHER, J A. (1949). The use of BAL (British Anti-Lewisite) in the treatment of the injurious effects of arsenic, mercury and other metallic poisons. *Ann. Intern. Med.*, **31**, 545–554.

MAGOS, L. & CLARKSON, T W. (1972). Atomic absorption determination of total, inorganic, and organic mercury in blood. *J. Assoc. Off. Anal. Chem.*, **55**, 966–971.

MAGOS, L. & STOYTCHEV, Ts (1969). Combined effect of sodium maleate and some thiol compounds on mercury excretion and redistribution in rats. *Br. J. Pharmac.*, **35**, 121–126.

ÖSTLUND, K (1969). Studies on the metabolism of methyl mercury and dimethylmercury in mice. *Acta pharmac. tox.*, **27**, suppl. 1.

SWENSSON, A. & ULFVARSON, V. (1967). Experiments with different antidotes in acute poisoning by different mercury compounds. *Int. Arch. Gewerbepath. Gewerbehyg.*, **24**, 12–50.

TEISINGER, J & SRBOVA, J. (1964). Effect of D-penicillamine on the urinary excretion of mercury and lead. *Prac. Lek.*, **16**, 433–435.

(Received October 31, 1975.
Revised December 11, 1975.)

Br. J. Pharmac. (1976), **56**, 485–490

STEREOSELECTIVE BINDING IN CARDIAC TISSUE OF THE ENANTIOMERS OF BENZETIMIDE, AN ANTIMUSCARINIC DRUG

J.A. GRAY,[1] H. LÜLLMANN, F. MITCHELSON[2] & G.-H. REIL
Department of Pharmacology, University of Kiel, Kiel, West Germany

1 Benzetimide, possessing two stable enantiomers, dexetimide and levetimide, has been investigated in guinea-pig atria with respect to its atropine-like action and its tissue distribution.

2 The antagonistic potency of dexetimide was found to be over 6000 times higher than that of levetimide, the pA_2 values being 9.82 and 6.0 respectively.

3 The tissue accumulation was investigated for both isomers in the concentration range from 1.5×10^{-9} M to 10^{-6} M yielding tissue to medium ratios (T/M) of between approximately 50 and 10. The highest values were found for the lowest concentrations. At any concentration investigated, dexetimide exhibited a higher uptake than the levoisomer.

4 The rate of uptake and washout of dexetimide was extremely slow, that of levetimide being considerably faster at equimolar concentrations. The same pattern held true for the onset and decline of the antagonistic action.

5 The high accumulation was found to be almost entirely due to unspecific binding. Even in the case of dexetimide the relative size of the receptor compartment could not be determined. The unspecific binding sites displayed a certain stereoselectivity but to a much lesser extent than the specific receptor binding sites.

Introduction

Benzetimide is an atropine-like compound which acts in both the peripheral and central autonomic nervous systems. It has been shown in both intact organisms and isolated organs that the antimuscarinic action of benzetimide is significantly greater than that of atropine (Janssen & Niemegeers, 1967; Janssen, Niemegeers, Schellekens, Demoin, Lenaerts, van Nueten, van Wijngaarden & Brugmans, 1971). Like atropine, benzetimide exists chemically as a racemic compound, possessing two optical isomers. However, whereas in the case of atropine these isomers spontaneously interconvert, both dexetimide and levetimide, the enantiomers of benzetimide, are stable in this respect.

Chemical studies using X-ray diffraction and other methods have revealed the S-configuration of the highly active dextro-enantiomer. This conformation coincides closely with the trans-conformation of acetylcholine, which is thought to be responsible for the muscarinic activity of the molecule (Spek, Peerdeman, van Wijngaarden & Soudijn, 1971). For the sake of clarity the active groups of the benzetimide molecule which resemble atropine are heavily marked in the structural formula shown in Figure 1.

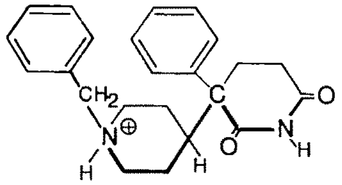
An earlier attempt was made to correlate the biological effect of atropine with its quantitative uptake and washout in guinea-pig atria. These investigations were hampered by the considerable degree of accumulation found in our radioactivity experiments, leading us to suspect the involvement of a large proportion of unspecific binding sites. However, these findings provided a possible explanation for the apparently paradoxical nature of atropine, with its competitive antagonistic action and its remarkably delayed uptake and washout. In order to see whether this kinetic behaviour held true for other antimuscarinic compounds, benzetimide has been subjected to similar investigations. We chose benzetimide since it seemed a particularly attractive tool: its enantiomers are not only stable but are also unusually dissimilar in their antagonistic potencies.

However, the results of our investigations led us in another direction. First, unusually high tissue to medium ratios (T/M) were obtained. Second, the unspecific binding sites appeared to be capable of exerting a certain stereoselectivity towards the two isomers. It is on these latter findings particularly that we wish to report in this paper.

---

[1] Present address: University College, Oxford.

[2] On sabbatical leave from: Victorian College of Pharmacy, 381 Royal Parade, Parkville, Victoria, Australia 3052.



Mol wt = 398,92
pA_2 = 9,82 Dexetimide
pA_2 = 6,0 Levetimide
pKa = 8,70

Figure 1 Structure of benzetimide. That part of the molecule, which resembles the active groups of atropine is accentuated (facing downwards). Mol. wt = molecular weight; pA_2 values denote the atropine like potency of the two enantiomers; pK_a = dissociation constant of the tertiary nitrogen.

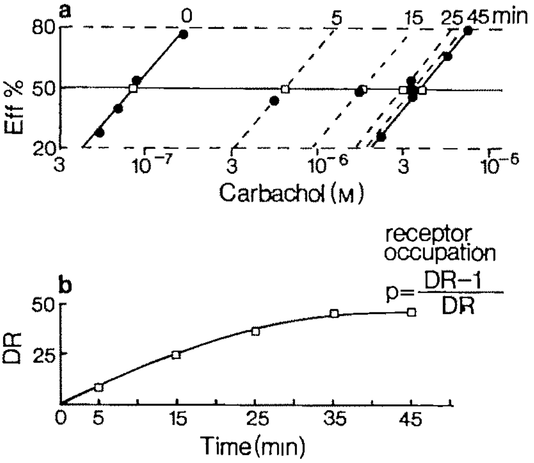


Figure 2 (a) Experimental procedure to demonstrate a parallel shift of the dose-response curve induced by levetimide (3×10^{-8} M) and the time course of onset of antagonistic action. (●) Experimental values; (□) calculated ED_{50}'s after addition of levetimide, as indicated on top of the curves. (b) Calculated dose-ratio values (DR) after adding the antagonist; for details see methods section.

Methods

Determination of the pharmacological effect of benzetimide

Electrically driven left auricles of guinea-pigs were used throughout this investigation. The following standard procedure was employed: after dissection the muscle was transferred to an organ bath containing Tyrode solution of the following composition (mmol/l): NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 12.0, NaH_2PO_4 0.21. The solution, with a resultant pH-value of 7.4, was gassed with 95% O_2 and 5% CO_2 and maintained at a constant temperature of 32°C. The stimulating pulses were of 4 to 5 ms duration, and 7 to 10 V, corresponding to double threshold; the frequency of stimulation was 180/minute. The contractions of each muscle were isometrically recorded via a standard transducer/amplifier/pen recorder system (SWEMA, Hellige 19).

After an equilibration period of at least 30 min the initial carbachol dose-response curve was determined. For this purpose, at least four different concentrations of carbachol, each eliciting an effect between 20 and 80% were applied, using the following procedure: (a) an equilibration period of 5 min in fresh Tyrode solution (b) exposure to a dose of carbachol for 3 min (c) a washout period of 2 minutes. This procedure having been carried out for each of the 4 concentrations of carbachol, the antagonist benzetimide was added to the Tyrode solution. Thereafter the time course of the onset of the antagonistic action was determined by applying test doses of carbachol at fixed time intervals. These doses were chosen to yield as closely as possible a 50% effect, as shown in Figure 2. After the attainment of final antagonistic equilibrium at which no further shift of the dose-response curve could be detected, a second complete dose-response curve for carbachol was determined. Fresh (benzetimide-free) Tyrode solution was then taken and the same procedure adopted to determine the disappearance, or decline of the antagonistic action.

Determination of the uptake and washout of ^3H-labelled benzetimide

Tritium-labelled dexetimide and levetimide were used, the specific activity amounting in both cases to 600 mCi/mmol. The radiochemical purity was determined as greater than or equal to 97% for dexetimide or less than or equal to 99% for levetimide. Throughout the experiments large organ baths containing electrodes capable of accommodating several auricles at once were used. After an equilibration period of 30 min in normal Tyrode solution the isolated auricles were transferred to a bath containing the radioactively labelled compound; 1.5×10^{-9} M was found to be the lowest concentration which could be reliably employed in practice, the Tyrode solution then yielding a disintegrations per minute (d/min) value of approximately 700/ml i.e. at least four times that of the background count. The auricles were removed from the bath after various fixed periods of time (5–180 minutes). Each was blotted according to a

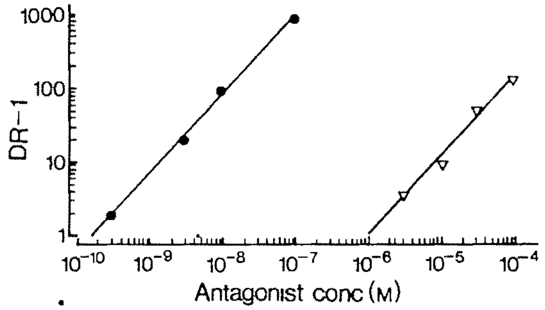


Figure 3 Double logarithmic plot (according to Arunlakshana & Schild, 1959) to demonstrate the competitive mode of antagonism, and the different antagonistic potencies of the two enantiomers. Dexetimide (●), $pA_2=9.82$. Levetimide (△), $pA_2=6.0$.

standard procedure, weighed and then dissolved in 1 ml of a tissue solvent (Soluene 50, Fa. Packard-Instruments). Aliquots from each sample of dissolved tissue were mixed with liquid scintillation fluid (Unisolve, Fa. Zinsser) neutralized by 0.1 M HCl, and counted in a scintillation counter (Packard Tri-Carb Liquid Scintillation Spectrometer, Model 544).

For the washout experiments, the auricles were loaded with the labelled benzetimide at different concentrations for 60 minutes. The tissue samples were then transferred to a bath containing 'benzetimide-free' solution and removed after various fixed periods of time. Thereafter the same procedure as that used for the uptake experiments was adopted.

The radioactivity counts were then expressed as d min^{-1} g^{-1} wet weight (w.wt.) or as tissue/medium ratios (d min^{-1} g^{-1} w.wt./d min^{-1} ml^{-1} of incubation medium).

A brief explanation of the dose-ratio (DR) technique (Paton, 1961)

Information concerning the initial slope of the curve and the concentration producing a 50% effect was obtained from the original dose-response curve. As shown in Figure 2 a parallel slope is evaluated at antagonistic equilibrium. From this fact we may assume a parallel shift of the dose-response curve throughout the onset of antagonistic action. Hence a theoretical ED_{50} can be calculated for the time of each test dose application. The ratio, ED_{50} at time t in the presence of antagonist/initial ED_{50}, is the dose-ratio (DR). According to Paton (1961) the receptor occupation, p, is defined as (DR−1)/DR. Using the p-values thus obtained, semilogarithmic plots can be made for the onset and decline of reactions as seen in Figures 4 and 5.

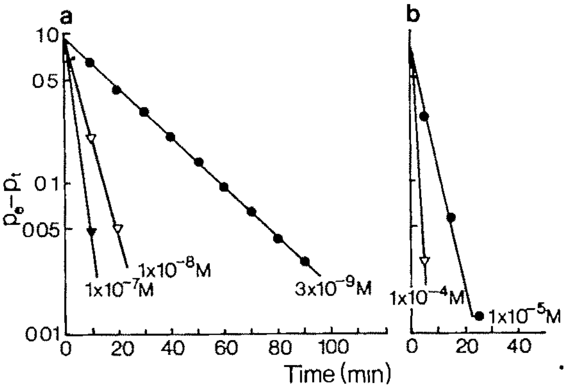


Figure 4 Onset of the antimuscarinic action of the two enantiomers in guinea-pig auricles Ordinates: receptor occupation (p_e-p_t); abscissae: time of incubation. (a) Half-life times of the onset of dexetimide's action were 18, 5 and 2 min for the concentrations 3×10^{-9} M, 1×10^{-8} M and 1×10^{-7} M respectively. (b) The corresponding values for levetimide were 4 and 1 min at concentrations of 1×10^{-5} M and 1×10^{-4} M, respectively The points represent means of at least 6 individual experiments, s e. mean was less than 5%

Results

Onset and decline of antagonistic action

When the dose-ratios obtained for the onset reaction were plotted against time a final equilibrium value was attained as shown in the lower part of Figure 2. The level of this final value was dependent upon the concentration of the antagonist used, that is to say the higher the concentration, the higher the level and the speed of its attainment.

As shown in Figure 3 a double logarithmic plot was then made of the equilibrium values (DR−1) versus the respective antagonistic concentrations. Straight-line graphs were obtained for both enantiomers. The negative logarithmic value of the abscissal cutting point of such a line is equal to the pA_2-value. The corresponding pA_2-value for dexetimide was found to be 9.82, that for levetimide 6.0. Thus the difference between the antagonistic potencies of the isomers is remarkably great, exceeding a factor of 6600 as already reported by Mitchelson (1975) and Soudijn, van Wijngaarden & Ariëns (1973).

In order to obtain information about the time course of the onset and decline of antagonistic action, graphs were plotted as shown in Figures 4 and 5. For onset (see Figure 4) receptor occupation at equilibrium, which is p_e, minus receptor occupation at time t which is p_t was plotted against time. In this way straight-line curves of negative slope could be

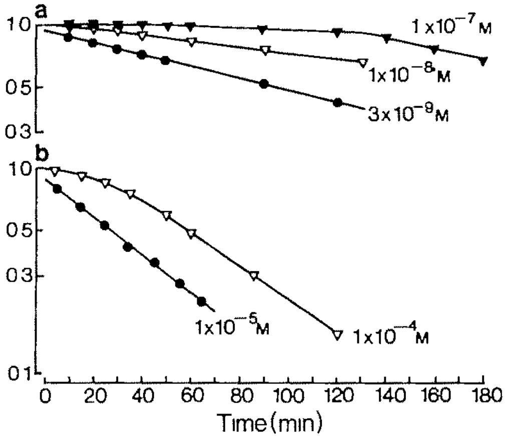


Figure 5 Decline of the antimuscarinic action of the two enantiomers in guinea-pig auricles: (a) dexetimide, (b) levetimide. Ordinates: receptor occupation (p), abscissae, time of washout of the compounds. Only the lowest concentrations of both enantiomers yielded straight lines with half-life times of 110 min for 3×10^{-9} M of dexetimide and 35 min for 1×10^{-5} M of levetimide, concentrations which are approximately equieffective with respect to the antagonistic action. The points represent means of at least 6 individual experiments; s.e. mean was less than 5%.

described, thus making possible the evaluation of half-life times for the time course of the onset reaction. The intercept with the ordinate represents the receptor occupation after the attainment of antagonistic equilibrium. For dexetimide it is 93% for a concentration of 3×10^{-9} M; 98% for 10^{-8} M and 99% for 10^{-7} M. For levetimide it is 90% for a concentration of 10^{-5} M, 98% at 10^{-4} M. Longer half-life times were found for lower concentrations. Nevertheless at equieffective concentrations of both isomers the half-life time of onset was considerably shorter in the case of levetimide.

To describe the decline of the reaction, graphs of receptor occupation (p) against time were plotted as shown in Figure 5. At low concentrations straight lines representing a monoexponential decline of the receptor occupation were found for both isomers. However, with increasing concentration a delay of the initial phase of the time course of decline became evident. This 'plateau' effect was most pronounced for dexetimide at the highest concentration used. This phenomenon will be discussed at a later stage. In comparing the decline curves of the two isomers at equieffective concentrations it could be seen that the decline in receptor occupation was significantly more rapid in the case of levetimide.

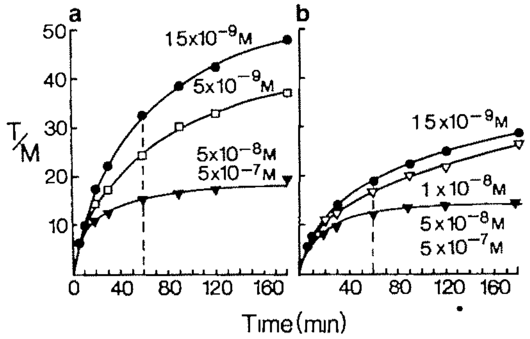


Figure 6 Accumulation of ^{3}H-labelled enantiomers in guinea-pig auricles: (a) dexetimide, (b) levetimide. Ordinates: concentration of the compounds expressed as tissue to medium (T/M) ratios. The points represent means of at least 8 individual auricles, s.e. mean was less than 5%. The dotted lines indicate the T/M values at which the washout experiments were started (see Figure 7)

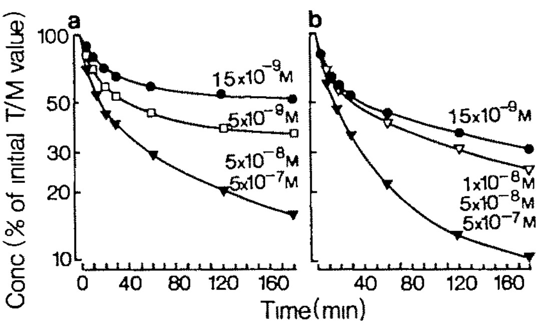


Figure 7 Release of ^{3}H-labelled enantiomers in guinea-pig auricles. Ordinates: concentration of drugs expressed as percentage of the initial T/M value after 60 min of incubation (see Figure 6); abscissae: time of washout. The points represent means of at least 8 individual auricles; s.e. mean was less than 5%.

Uptake and washout of labelled benzetimide

The uptake of the two isomers was investigated in the concentration range, 1.5×10^{-9} M to 10^{-6} M. As depicted in Figure 6 graphs of T/M ratios against time were plotted with the following results: first after 3 h of incubation in no case was a final equilibrium value attained; second the highest T/M ratios corresponded to the lowest concentrations of both isomers, e.g. T/M values of 48, 28 for 1.5×10^{-9} M dexetimide and levetimide respectively. These values reflect a surprisingly high accumulation capacity in the cardiac

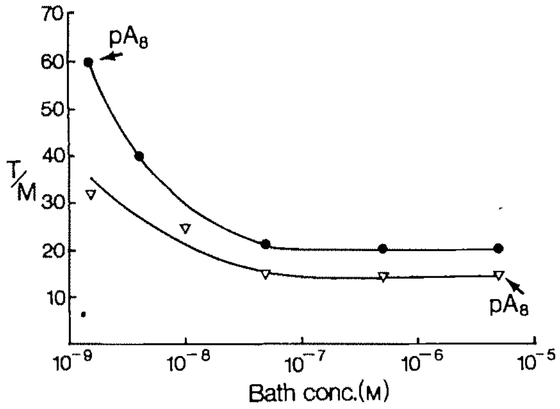


Figure 8 The final values of accumulation of the isomers are plotted versus the corresponding bath concentrations. (●) Dexetimide; (▽) levetimide. The antimuscarinic activity is marked by the pA$_8$-values for both enantiomers

tissue. At higher concentrations the T/M values diminished and reached a minimum at a concentration of 5×10^{-8} M. Above this concentration no further decline in the T/M values was detectable (T/M values of 20, 15 for dexetimide, levetimide respectively). A more detailed analysis of the curves revealed that in no case was a simple monoexponential function obeyed. Furthermore, the rate of accumulation was proved to be consistently higher for levetimide.

The washout curves were plotted semi-logarithmically in relative values as shown in Figure 7. The T/M values after 60 min of incubation (see dotted lines in Figure 6) were taken as the 100% values or starting points for the washout process. In general the drug release process could be described in terms of an initial fast and second slower phase. With increasing concentration the process was accelerated. As can be clearly seen in Figure 7, at equal concentrations the washout of levetimide is quicker than that of the dextro-isomer.

Discussion

As has already been mentioned the advantages of using benzetimide in these kinetic studies stem from the stability of its enantiomers and the unusually large difference between their antagonistic potencies (see Figure 3). The combination of both these properties provides us with the possibility of distinguishing clearly between the two enantiomers in our investigations. This distinction may be applied to both static equilibrium values and kinetic data obtained. This is clearly demonstrated in respect of the former in Figure 8.

Differing final T/M values were found for the two isomers after a 3 h incubation period. It should be noted that no terminal equilibrium values were obtained. Figure 8 shows the relationship between the calculated equilibrium T/M ratios and the corresponding bath concentrations. This represents the equilibrium binding curves of both isomers over the entire concentration range investigated. On examination of the diagram the following become clear: (1) that the overall binding differs between the two isomers; (2) that below a concentration of 10^{-7} M a saturable compartment is evident in the case of both isomers (i.e. the curves show a negative gradient); (3) that above a concentration of 10^{-7} M a non saturable compartment is present (i.e. beyond this concentration the curves run parallel to the abscissa scale). These results should be analysed in conjunction with the kinetic data obtained from our onset/decline experiments and the corresponding uptake/washout investigations. Such an approach reveals the following: dexetimide attains approximately 100% receptor occupation by a concentration of 10^{-7} M (see Figure 4). In contrast, at such a concentration there is no measurable receptor occupation for levetimide ($pA_2 = 6{,}0$). In this case the total receptor occupation corresponds to a concentration exceeding 10^{-5} M. Relating this to the binding curves in Figure 8 an interpretation can be made as follows: whereas both enantiomers show an initial 'saturable' phase, different binding sites are involved at this stage. In the case of dexetimide this is the receptor compartment; in the case of levetimide we must deduce that an unspecific saturable binding compartment is responsible, displaying nevertheless a higher affinity for the drug than the specific compartment. Indeed when the receptor compartment of levetimide eventually comes into play it is overwhelmed by a predominant unspecific unsaturable compartment.

The difference between the initial phases of the two curves can thus be understood in terms of the presence of two distinct saturable binding sites.

During the later 'unsaturable' phase of accumulation, when physico-chemical properties are normally assumed to underly the distribution of a drug, one would expect the T/M values for the two isomers to reach the same final level. However, it should be noted that this is not the case. In addition the results of our radioactivity experiments reveal significantly differing rates of uptake and washout when comparing the behaviour of both isomers at equal concentrations. Combining these two results we may justifiably conclude that unspecific binding sites may also display stereoselective properties, being capable of distinguishing between left and right rotating enantiomers.

At present little information is available in the literature concerning the stereo-selectivity of unspecific binding sites. Different binding affinities of plasma albumin have been demonstrated for the enantiomers of benzodiazepines by Müller & Wollert (1975) and for those of noradrenaline by Powis (1975). An investigation of the binding of the isomers of noradrenaline in connective tissue (Powis, 1973) and in heart and brain tissue (Iversen, Jarrott & Simmonds, 1971) disclosed a certain stereoselectivity also of the unspecific binding. With respect to benzetimide, the detailed study of Beld & Ariëns (1974), using a membrane fraction of smooth muscle and brain tissue, did not take into account a possible stereoselectivity of unspecific binding sites. A useful chemical procedure was evaluated by Blaschke (1972); by using polyacrylamides substituted by either left or right rotating substances, racemic compounds could be separated by this mode of column chromatography.

A further conclusion may be drawn from the results shown in Figure 8. As has already been stated, the binding curve for levetimide exhibits a negative slope at a concentration range corresponding to the filling of only a saturable unspecific compartment. It is therefore possible that the corresponding negative slope for dexetimide is due to the filling of not only a specific but also an unspecific compartment. In addition, from the negative diverging slopes it is not possible to determine the degree of stereoselectivity shown towards the two isomers. Higher affinity to the tissue binding sites shown at the lower concentration range may parallel an increasing ability to discriminate between the two isomers, which cannot be quantitatively determined. Thus a reliable estimation of the size of the receptor compartment in the case of dexetimide is not possible.

The decline curves depicted in Figure 5 reveal that an initial 'plateau' phase is present at higher concentrations of both isomers. This may be interpreted as reflecting a high initial release of the drug predominantly from the unspecific binding sites. Thus a relatively high antagonist concentration is kept in the biophase, thereby delaying the release of the drug from the receptor during this initial phase. Thereafter, when the concentration of antagonist in the biophase falls to a critical level, the release from the receptor obeys a monoexponential function. The 'plateau' may thus be explained in terms of an overwhelming initial contribution to the diffusion process by the unspecific binding sites. From this phenomenon it is clear that unspecific binding sites may be closely involved in the kinetic behaviour of benzetimide.

The quantitative nature of this phenomenon is at present under investigation together with the mechanism underlying the unusually high tissue accumulation of benzetimide.

We acknowledge the expert technical assistance of Ms E. Obst. This work was supported by the Deutsche Forschungsgemeinschaft, Bonn Bad Godesberg. The labelled compounds were kindly supplied by Drs W. Soudijn and J. van Wijngaarden (Janssen, Pharmaceutica, Beerse, Belgium).

References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmac. Chemother.*, **14**, 48–58.

BELD, A.J. & ARIENS, E.J. (1974). Stereospecific binding as a tool in attempts to localize and isolate muscarinic receptors. Part II. Binding of (+)- and (–)-benzetimide and atropine to a fraction from bovine tracheal smooth muscle and to bovine caudate nucleus. *Eur. J. Pharmac.*, **25**, 203–209.

BLASCHKE, G. (1972). Chromatographische Racemattrennungen. Habilitationsschrift, Kiel, 1972.

IVERSEN, L.L., JARROTT, B. & SIMMONDS, M.A. (1971). Differences in uptake, storage and metabolism of (+)- and (–)-noradrenaline. *Br. J. Pharmac.*, **43**, 845–855.

JANSSEN, P.A.J. & NIEMEGEERS, C.J.E. (1967). The peripheral and central anticholinergic properties of benzetimide (R 4929) and other atropine like drugs as measured in a new anti-pilocarpine test in rats. *Psychopharmacologia*, **11**, 231–254.

JANSSEN, P.A.J., NIEMEGEERS, C.J.E., SCHELLEKENS, K.H.L., DEMOEN, P., LENAERTS, F.M., VAN NUETEN, J.M., VAN WIJNGAARDEN, J. & BRUGMANS, J. (1971). Benzetimide and its optical isomers. *Drug Res.*, **21**, 1365–1373.

MITCHELSON, F. (1975). The antimuscarinic action of an alkane-bis-ammonium compound alone and in combination with (+)-benzetimide in the electrically stimulated guinea pig atrium. *Eur. J. Pharmac.*, **33**, 237–246.

MÜLLER, W.E. & WOLLERT, U. (1975). High stereospecificity of the benzodiazepine binding site on human serum albumin. *Mol. Pharmac.*, **11**, 52–60.

PATON, W.D.M (1961). A theory of drug action based on the rate of drug receptor combination. *Proc. roy. soc. Ser. B*, **154**, 21–69.

POWIS, G. (1973). Binding of catecholamines to connective tissue and the effect upon the responses of blood vessels to noradrenaline and to nerve stimulation. *J. Physiol., Lond.*, **234**, 145–162.

POWIS, G (1975). The binding of catecholamines to human serum proteins. *Biochem. Pharmac.*, **24**, 707–712.

SOUDIJN, W., VAN WIJNGAARDEN, J & ARIENS, E.J. (1973). Dexetimide, a useful tool in acetylcholine-receptor localization. *Eur. J. Pharmac.*, **24**, 43–48.

SPEK, A.L., PEERDEMAN, A.F., VAN WIJNGAARDEN, J. & SOUDIJN, W. (1971). The absolute configuration of crystal structure of the anticholinergic drug dexbenzetimide. *Nature, Lond.*, **232**, 575–576.

(Received November 14, 1975)

Br. J. Pharmac. (1976), 56, 491–493

EFFECT OF SAS (A NEW 10-N-ACYLAMINOPHENOTHIAZINE) ON GASTRIC SECRETION AND ULCERATION IN RATS

A. KUMAR, H.L. SHARMA & V.N. SHARMA[1]
Department of Pharmacology, S.M.S. Medical College, Jaipur, India

The antiulcer and antisecretory activity of 2-chloro-10-[4'(*N*-β-hydroxyethyl) piperazinyl-1'] acetylphenothiazine (SAS) has been investigated. At 10 and 20 mg/kg (s.c.) the drug was found to possess potent antigastric secretory and antiulcer activity (both in shay and stress ulcers) and did not exhibit any peripheral parasympathetic blocking activity. The pronounced antispasmodic activity of SAS was nonspecific rather than a specific parasympatholytic effect.

Introduction Most of the antiulcer drugs in use today, lack specificity of action and efforts continue to find new antiulcer drugs devoid of undesirable secondary effects. Amongst the phenothiazines, chlorpromazine in high doses (20 mg/kg) has been reported to possess antigastric secretory and antiulcer activity (Radwan & West, 1971). However, at such high doses, many other effects are produced and the antiulcer activity is not specific. Secergan, a new quaternary acylamino phenothiazine derivative, has been reported to be an effective antiulcer and antispasmodic drug (Tomenius, 1957). However, quaternary compounds are less predictable in action than tertiary analogues (Anichkov & Zavodskaya, 1968) and introduction of a tertiary amine function in the side chain, has yielded compounds which produce selective parasympathetic blockade (Pardo, Vargas, Cato & Laguna, 1956). Hence on theoretical grounds, acylaminophenothiazines, with a tertiary amino group in the side chain, should be preferable to quaternary compounds like Secergan. With this in view, a series of 10-*N*-acylaminophenothiazines were synthesized (Sharma, Banerjee, Sharma & Mital, 1970) and the present communication deals with the observed antigastric secretory and antiulcer activity of one of these compounds *viz* 2-chloro-10-[4'-(*N*-β-hydroxyethyl) piperazinyl-1'] acetylphenothiazine (SAS).

Methods The gastric antisecretory effect of SAS was determined by the method of Meyer, Cummings, Bass & Collier (1965) in 48 h fasted, 4 h ligated rats of either sex (180–200 g) of the Haffkin strain. The test compound, dissolved in distilled water was given subcutaneously immediately after ligation to groups of 10 rats. Four hours after ligation, the rats were killed and the stomach contents were removed and centrifuged, at 2000 rev/min for 10 min and subsequently were assessed for volume, pH and total acidity. The samples (0.1 ml) of gastric juice were titrated against 0.01N NaOH using phenolphthalein as indicator. The results were expressed in mEq/l of total acids.

Two methods for producing gastric lesions in rats were used: (a) Temporary pylorus ligation: groups of ten female rats (120–150 g) were ligated at the pyloric sphincter by the method of Shay, Komarov, Fels, Merange, Gruenstein & Sipiet (1945) as modified by Yasuo Ishi (1969). Briefly, rats were housed individually and fasted for 24 h and the pylorus ligated under light ether anaesthesia. After suturing the wound, SAS or an equivalent volume of 0.9% w/v NaCl solution (saline) was injected subcutaneously. In one group, SAS was also administered orally at a dose of 20 mg/kg. After 18 h, the mortality in the groups, if any, was noted and then the stomachs of all the surviving rats were removed, cut along the greater curvature, and examined visually for the degree of ulceration using a score from 1 to 5 depending upon severity of ulceration (Adami, Marazzi-Uberti & Turba, 1964). Mean ulcer score was then calculated from the individual scores. (b) Immobilization of rats (Adami *et al.*, 1964): rats of either sex, fasted for 24 h, were immobilized by wrapping them tightly with a flexible wire mesh sheet. The test compound or control vehicle was injected subcutaneously 30 min before immobilization and 12 h after the first dose. After 24 h of immobilization, stomachs were removed and gastric mucosal damage was assessed as described for the Shay rats.

The antispasmodic activity was tested on rabbit isolated ileum preparations. Tyrode solution containing 1% barium chloride was used to maintain a constant spasm of the ileum. The spasmolytic activity of SAS, was compared with papavarine as reference standard in a series of six experiments.

The spasmolytic, antiacetylcholine and antihistamine activity of SAS was studied on the guinea-pig isolated ileum preparation allowing a 2 min drug contact time. Barium chloride (2 mg/ml), acetylcholine (1 μg/ml) and histamine (10 μg/ml) were used as spasmogens and activities were compared with papavarine hydrochloride, atropine sulphate and diphenhydramine respectively. The ED_{50}

---

[1] Present address: Department of Pharmacology, S.N. Medical College, Jodhpur, India.

for SAS as well as for the standard drugs was calculated by graphic interpolation and the activity ratios, with respect to the appropriate agonists, were calculated.

The effects of SAS on blood pressure, respiration and on intact intestine was studied on dogs anaesthetized with pentobarbitone sodium (30 mg/kg). Up to 20 mg/kg of the test compound was used to study its antagonism to acetylcholine, adrenaline, noradrenaline, isoprenaline, histamine and dimethylphenylpaperazinium iodide (DMPP). The effect of a 1% solution of SAS on iris muscle was also studied in a group of six rabbits and compared with that of 0.1% atropine sulphate.

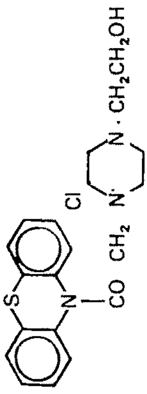
Results and Discussion SAS in doses of 10 mg/kg and 20 mg/kg, greatly reduced the volume and total acidity of gastric secretion in 4 h pylorus ligated rats. A significant increase in pH of the gastric contents of drug-treated rats was also observed (Table 1).

SAS in doses up to 5 mg/kg intravenously, was found to have no effect on the blood pressure and respiration of anaesthetized dogs. However, a dose-dependent, transient fall in blood pressure was observed at 50 mg/kg, the blood pressure recovering in less than one minute. Doses of 20 mg/kg SAS did not alter the blood pressure responses elicited by acetylcholine, histamine, noradrenaline, adrenaline, isoprenaline and DMPP. A 1% solution of the test compound did not show any mydriatic activity in the rabbit eye.

The gastric anti-secretory activity of SAS is not due to peripheral parasympathetic blockade, as the drug even in large doses does not antagonize the blood pressure responses to acetylcholine and DMPP and elicits no mydriatic activity. Failure to block the compensatory reflexes triggered by the stimulation of carotid and aortic baroreceptors also indicate that the drug has no parasympatholytic activity. Although several compounds, which are devoid of any anti-acetylcholine or ganglion blocking activity, are known to possess antisecretory action, the exact mechanism of such a non-specific action is not clear. SAS is structurally analogous to chloracizine, a phenothiazine antidepressant (Lappin & Shchelkunov, 1963), and indeed antidepressants like imipramine possess antisecretory effects. The antidepressant effects of SAS have recently been observed (unpublished data) but whether this is responsible for the observed antisecretory activity is debatable.

In Shay rats, gastric lesions were observed mainly in the rumenal area and the control group showed 35% mortality. However, in SAS-treated Shay rats, the mortality (25%) and severity of ulcers were greatly reduced even at 10 mg/kg ($P < 0.05$). The protection against mortality (10%) and gastric mucosal damage was more marked at 20 mg/kg subcutaneously while at 20 mg/kg orally the mortality was nil and the mean

Table 1 Effect of 2-chloro-10-[4'(N-β-hydroxyethyl) piperazinyl-1'] acetylphenothiazine (SAS) on gastric secretion, total acidity, pH and ulceration in rats‡



Dose mg/kg s.c.	Mean volume ±s.e.	Total acidity ±s.e.	pH ±s.e.	% Ulceration Shay rats	% Ulceration Restrained rats	Mean Ulcer Score ±s.e. Shay rats	Mean Ulcer Score ±s.e. Restrained rats	% Mortality Shay rats	% Mortality Restrained rats
Control	5.8±1.2	68.1±13.7	2.1±0.35	85	75	4.3±0.5	3.8±0.3	35	—
5	4.0±0.3	48.2±16.5	3.6±0.2**	60	—	3.1±0.3	—	25	—
10	2.2±0.7**	18.4±4.7**	4.0±0.1***	22	40	2.1±0.4**	1.4±0.3**	25	—
20	1.1±0.2***	13.1±2.4***	5.8±0.2***	12	15	1.1±0.1***	0.6±0.2***	10	—
20*	—	—	—	15	—	0.8±0.2***	—	Nil	—

‡ each group contained 10 rats; * Given orally; ** $P < 0.05$, *** $P < 0.01$

ulcer score was only 0.8 ± 0.2 ($P < 0.01$). This protection offered by SAS against ulceration and mortality, could be due to its effect in reducing volume and acidity of gastric secretion. The local anaesthetic activity of SAS (Sharma, Mital, Banerjee & Sharma, 1971) might also have played some role in this protective action, either by blocking gastrin release from the gastric antrum or by reducing ulcer pain (Posey, Boler & Posey, 1969). However, conclusive evidence on this point is lacking at this stage.

The preventive effect of SAS against the severity and incidence of immobilization stress-induced gastric lesions was significant ($P < 0.01$) at 20 mg/kg subcutaneously (Table 1). Immobilization results in ulcers which are mainly neurogenic in origin. Hence the protective effect of SAS against restraint-induced ulcers indicate that its anti-secretory effect may not be the sole factor responsible for its antiulcer activity. Some central action might also be involved. Although at effective dose levels, peripheral parasympatholytic or sympatholytic activities are lacking in SAS, the exact mechanism for the observed antiulcer activity could not be ascertained.

The test compound produced a general spasmolytic effect as it reversibly antagonized acetylcholine, histamine and $BaCl_2$-induced contractions of the guinea-pig isolated ileum. The ED_{50}, which inhibits maximum contraction by 50%, was found for SAS to be 0.9 µg/ml, 0.6 µg/ml and 8 µg/ml compared with the ED_{50} of standard antagonists: 81 ng/ml (diphenhydramine) 4 ng/ml (atropine) and 3.5 µg/ml (papavarine) respectively. On the rabbit isolated ileum it produced relaxation and inhibited pendular movements. In the intact intestine, in anaesthetized dogs, prolonged relaxation was observed on intravenous administration (2 mg/kg and more) of SAS.

The pronounced antispasmodic effects of SAS are consistent with reports that acylamino phenothiazines (Millot & Guillot, 1965) and some other 10-*N*-esteralkylaminophenothiazines (Dahlbom & Ekstrand, 1952) possess spasmolytic activities. Here again, the observed antispasmodic action does not seem to be a specific parasympatholytic effect as the antagonistic action of the drug against $BaCl_2$-induced spasm is also pronounced. This musculotropic action could also be due to its local anaesthetic activity (Sharma *et al.*, 1971) which may cause a nonspecific relaxant action, though several antispasmodics like adiphenine and amprotropin which possess local anaesthetic properties have no demonstrable effects on gastric secretion.

Our findings are therefore consistent with the views expressed by Gordon (personal communication, 1970) as well as by Anichkov & Zavodskaya (1968) that nonquaternary antiulcer and antisecretory agents should be preferable to the quaternary ones because of their lesser peripheral cholinergic involvement.

References

ADAMI, E., MARAZZI-UBERTI, E. & TURBA, C. (1964). Pharmacological research on gefarnate, a new synthetic isoprenoid with an antiulcer action. *Arch. int. Pharmacodyn*, **147**, 113–145.

ANICHKOV, S.V. & ZAVODSKAYA, I.S. (1968). *The experimental basis of gastric ulcer pharmacotherapy.* Oxford: Pergamon Press.

DAHLBOM, P. & EKSTRAND, T. (1952). 10-Aminoacyl phenothiazines-II. Aminobutyryl derivatives. *Acta chem. scand.*, **6**, 1285–88.

LAPPIN, I P. & SHCHELKUNOV (1963). Amphetamine induced changes in behaviour of small laboratory animals as simple tests for evaluation of central effects of new drugs; *Proc. 2nd Int. Pharmacological Meeting*, Prague, Aug. 20–23, Vol. I, pp. 205–215. London: Pergamon Press.

MEYER, R.F., CUMMINGS, B.L., BASS, P & COLLIER, H.O.J. (1965). Synthesis and pharmacological action of 3-amino-2, 1-benzisothiazoles. *J. med. Chem.*, **8**, 515–19.

MILLOT, L. & GUILLOT, J (1965). French Patent 2659, through *Chem. Abstr.*, **62**, 570.

PARDO, E.G., VARGAS, R., CATO, J. & LAGUNA, J. (1956). Pharmacologic actions of ethyl-methyl isooctenylamine (EMOA), a new parasympathetic ganglionic blocking agent. *J. Pharmac. exp. Ther.*, **116**, 377–86.

POSEY, E L (Jr.), BOLER, K. & POSEY, L. (1969). Inhibition of food stimulated gastrin release by a topical anaesthetic, oxethazine. *Am. J. Digest. Diseases*, **14**, 797–804.

RADWAN, A G. & WEST, G.B. (1971). Effect of aminoquanidine, chlorpromazine and NSD-1055 on gastric secretion and ulceration in the shay rat. *Br. J. Pharmac.*, **41**, 167–69.

SHARMA, H.L., BANERJEE, S.P., SHARMA, V.N. & MITAL, R.L. (1970). A possible confirmation acquired by phenothiazine drugs while interacting with CNS receptor sites. *Ann. Soc. Sci. Brux.*, **84**, 55–68.

SHARMA, V N., MITAL, R L, BANERJEE, S.P. & SHARMA, H.L. (1971). Phenothiazines as local anaesthetics. *J. med. Chem.*, **14**, 68–70.

SHAY, H., KOMAROV, B A., FELS, S S., MERANGE, D., GRUENSTEIN, M. & SIPIET, H. (1945). A simple method for the uniform production of gastric ulceration in the rat. *Gastroenterology*, **5**, 43–61.

TOMENIUS, J. (1957). Clinical trials of 10- (α-dimethylaminopropionyl) phenothiazine methobromide. *Acta med. scand.*, **157**, 11–22.

YASUO ISHI (1969). Critical studies on pylorus ligated rat (Shay-rat). *Jap. J. Pharmac.*, **19**, 125–133.

(Received October 29, 1975.)

Br. J. Pharmac. (1976), **56**, 494–496

HISTAMINE H_2-RECEPTORS IN THE HUMAN PERIPHERAL CIRCULATION

P. CHIPMAN & W.E. GLOVER

School of Physiology and Pharmacology, University of New South Wales, PO Box 1, Kensington, New South Wales, Australia 2033

Histamine (10 µg/min for 3 min) infused into the brachial artery caused an increase in forearm blood flow which was reduced by mepyramine (25 mg). This effect was most marked in the first minute of the infusion. Metiamide (25 mg) had no effect on the dilatation during the infusion but caused a quicker return of flow to the resting level. The response was abolished when both drugs were given in combination. It is concluded that the response is initiated mainly by stimulation of H_1-receptors and maintained by H_1- and H_2-receptors; continued activity of H_2-receptors may account for the slow return of flow to the pre-infusional level.

Introduction Responses to histamine are mediated by mepyramine-sensitive H_1-receptors or burimamide- and metiamide-sensitive H_2-receptors (Black, Duncan, Durant, Ganellin & Parsons, 1972). Both types of receptor are involved in cardiovascular responses to histamine.

In the rabbit, the change in arterial pressure produced by an intravenous injection of histamine represents a balance between H_1-mediated pressor and H_2-mediated depressor components (Parsons & Owen, 1973; Carroll, Glover & Latt, 1974), and both H_1-vasoconstrictor and H_2-vasodilator receptors have been demonstrated in the same isolated artery (Parsons & Owen, 1973; Glover, Carroll & Latt, 1973). In contrast, both H_1- and H_2-receptors are involved in the vasodepressor response to histamine in the dog and cat. In these species, the response to histamine is partly reduced by mepyramine, and while it is unaffected by burimamide alone, it is abolished when both drugs are given in combination (Black *et al.*, 1972; Parsons & Owen, 1973; Powell & Brody, 1973).

Stimulation of H_1-receptors causes vasodilatation in man, since intra-arterial histamine produces an increase in forearm blood flow which is antagonized by mepyramine (Duff & Whelan, 1954). The role of H_2-receptors has not yet been established, although their activity has been demonstrated in isolated temporal arteries (Glover *et al.*, 1973), and the facial flushing observed during an intravenous infusion of histamine is blocked only when both mepyramine and burimamide are given (Wyllie, Hesselbo & Black, 1972). We now present evidence that both H_1- and H_2-receptors mediate the vasodilator response to histamine in the human forearm.

Methods Blood flow through the forearm was measured in 10 healthy student volunteers by venous occlusion plethysmography (Greenfield, 1954). Isotonic saline (0.9% w/v NaCl solution) was infused at a rate of 4 ml/min through a catheter in the brachial artery; drugs were added to the saline so that the dose for 1 min was contained in 4 ml. Two control infusions of histamine (10 µg/min for 3 min) were given at 10–15 min intervals. The infusions of histamine were repeated twice during the continuous infusion of either mepyramine maleate (5 experiments) or metiamide (5 experiments). The second antagonist was then added to the constant perfusion of saline and two final three min infusions of histamine were given, one at the rate of 10 µg/min and the other 20 µg/minute. The drugs used were histamine acid phosphate (D.G. Bull Laboratory), metiamide (Smith Kline & French) and mepyramine maleate (May & Baker).

Results The results are summarized in Figure 1. Before the administration of an antagonist, histamine caused an immediate and sustained increase in flow followed by a slow return towards the resting level when the infusion ended.

In 5 experiments (Figure 1a) mepyramine 25 mg was infused over 5 min and then given continuously at the rate of 0.25 mg/minute. The response to histamine was reduced but not abolished after 25 mg of mepyramine, and was not reduced further after another 12–15 min of infusion at 0.25 mg/min (3–5 mg of mepyramine). In particular, mepyramine altered the time course of the response. The onset of the vasodilatation was delayed by about 20 s and the average increase during the first minute was 25% of the control response, although it reached a plateau of 49% of the control response during the third minute.

In the other 5 experiments (Figure 1b) metiamide (1 mg/min) was infused continuously and the response to histamine tested after 10 and 25 minutes. Metiamide alone had no effect on the vasodilatation during the histamine infusion, but had a significant effect on the post-infusional dilatation; in the presence of metiamide flow returned to the pre-infusional level in the second minute after the infusion whereas in the third minute after the control infusion flow was still 45% above the resting level. This reduction of the post-infusional vasodilata-

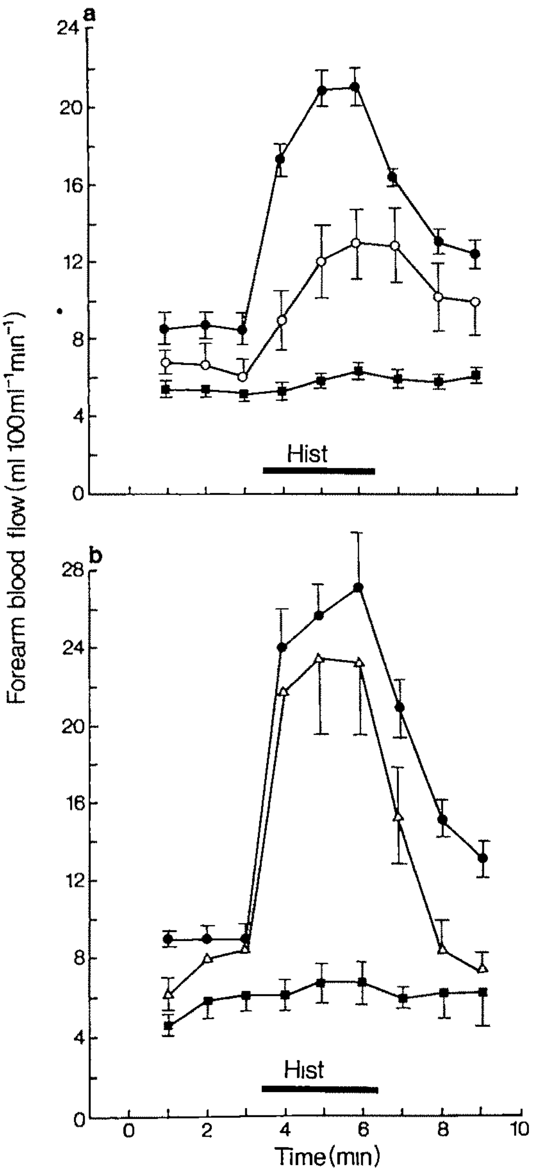


Figure 1 Effect of histamine (10 µg/min for 3 min) on forearm blood flow. Each point is the mean of 3 blood flow rates recorded in each min during 1–2 infusions in each of 5 subjects. Vertical bars show s.e. mean. (●) Control response (2 infusions in each subject); (a) in presence of mepyramine (○) (average of response to 2 infusions, the first after 25 mg, the second after a further 3–5 mg of mepyramine); (b) in presence of metiamide (△) (average of response to 2 infusions, the first after 10 mg, the second after a further 15 mg of metiamide); (■) in presence of both drugs in combination ((a) after mepyramine 30–35 mg and metiamide 10–15 mg; (b) after metiamide 30–40 mg and mepyramine 25 mg).

tion was seen after 10 mg of metiamide, and the response was not altered further after another 15 mg of metiamide.

In all 10 experiments the responses to histamine (10 µg/min for 3 min) and to a further infusion of 20 µg/min for 3 min, were completely abolished when both drugs were given in combination.

Discussion The results show that the vasodilator response to histamine in the human forearm which is only partially attenuated by a large dose of mepyramine is completely abolished when both mepyramine and metiamide are given in combination. It is therefore concluded that the response is mediated by both H_1- and H_2-receptors.

While mepyramine caused a significant reduction in all phases of the response to an infusion of histamine, its effect was most marked in the first minute. Metiamide alone had no effect on the vasodilatation during the infusion but in its presence flow returned more quickly than usual to the pre-infusional level. This suggests that there may be a difference in the time-course of the contributions made by the two types of receptor. The vasodilatation appears to be initiated mainly by stimulation of H_1-receptors and maintained by both H_1- and H_2-receptors. A more prolonged response to stimulation of H_2-receptors may account for the slow return to the pre-infusional level.

Although histamine release has been demonstrated following arterial occlusion and muscular exercise (Anrep, Barsoum, Salama & Souidan, 1944), it has been concluded that histamine does not play a significant role in reactive or post-exercise hyperaemia, since these responses are unaffected or only slightly reduced by antihistamines such as mepyramine (Duff, Patterson & Whelan, 1955). With our present knowledge, this conclusion can only be applied to histamine H_1-receptors and we are now investigating the possibility that H_2-receptors may make a contribution.

The work was carried out in the Vascular Laboratory at St Vincent's Hospital, Sydney. We thank the student volunteers and Sister M. Binnie for her assistance. We are grateful to Dr W.A.M. Duncan of Smith, Kline & French Ltd. for generous supplies of metiamide, and to Dr S. Kalowski of May & Baker (Australia) Ltd. for the mepyramine.

References

ANREP, G.V., BARSOUM, G.S., SALAMA, S. & SOUIDAN, Z. (1944). Liberation of histamine during reactive hyperaemia and muscle contraction in man. *J. Physiol., Lond.*, **103**, 297–305.

BLACK, T.W., DUNCAN, A.M., DURANT, C.T., GANELLIN, C.R. & PARSONS, E.M. (1972). Definition and antagonism of histamine H_2-receptors. *Nature, Lond.*, **236**, 385–390.

CARROLL, P.R., GLOVER, W.E. & LATT, N. (1974). Cardiovascular histamine receptors in the rabbit. *Aust. J. exp. Biol. med. Sci.*, **52**, 577–582.

DUFF, F., PATTERSON, G.C. & WHELAN, R.F. (1955). The effect of intra-arterial antihistamines on the hyperaemia following temporary arrest of the circulation in the human forearm. *Clin. Sci.*, **14**, 267–273.

DUFF, F. & WHELAN, R.F. (1954). The effects of antihistamine substances on the response to histamine of the blood vessels of the human forearm. *Br. J. Pharmac. Chemother.*, **9**, 413–418.

GLOVER, W.E., CARROLL, P.R. & LATT, N. (1973). Histamine receptors in human temporal and rabbit ear arteries. In *International symposium on histamine H_2-receptor antagonists*, ed. Wood, C.J. & Simkins, M.A., pp. 169–174. Welwyn Garden City: Smith Kline & French Laboratories Limited.

GREENFIELD, A.D.M. (1954). A simple water-filled plethysmograph for the hand or forearm with temperature control. *J. Physiol., Lond.*, **123**, 62P.

PARSONS, M.E. & OWEN, D.A.A. (1973). Receptors involved in the cardiovascular responses to histamine. In *International Symposium on Histamine H_2-receptor Antagonists*, ed. Wood, C.J. & Simkins, M.A., pp. 127–135. Welwyn Garden City: Smith Kline & French Laboratories Limited.

POWELL, J.R. & BRODY, M.J. (1973). Identification of two vascular histamine receptors in the dog. In *International Symposium on Histamine H_2-receptor Antagonists*, ed. Wood, C.J. & Simkins, M.A., pp. 137–146. Welwyn Garden City: Smith Kline & French Laboratories Limited.

WYLLIE, J.H., HESSELBO, T. & BLACK, J.W. (1972). Effects in man of histamine H_2-receptor blockade by burimamide. *Lancet*, **ii**, 1117–1120.

(Received October 20, 1975)

INDEX TO VOLUME 56

AUTHOR INDEX

A

ABAITEY, A.K & PARRATT, J.R. Cardiovascular effects of diethylcarbamazine citrate, 219

ABU-MURAD, C, GRIFFITHS, P.J. & LITTLETON, J.M (introduced by BROWNLEE, G.) Catecholamine metabolism and liver dysfunction during induction of ethanol dependence, 377P

ADAMS, M.D., CHAIT, L.D. & EARNHARDT, J.T. Tolerance to the cardiovascular effects of Δ^9-tetrahydrocannabinol in the rat, 43

ADEBANJO, A.O., AMBACHE, N & VERNEY, J. The inhibitory transmission to the internal anal sphincter, 392P

ALPS, B.J., DEVOY, P.W & WATERFALL, J.F. The evaluation of the novel pressor activity of γ-piperidinobutyramide (WY 20051, DF480), 179

AMADUCCI, L., MANTOVANI, P. & PEPEU, G. Effect of some phospholipids on acetylcholine output from the cerebral cortex in the rat, 379P

AMBACHE, N. & KILLICK, S.W. Species variation in motor transmission to the retractor penis muscle, 351P

AMBACHE, N. *see* ADEBANJO, A.O., 392P

ANDERSON, A.A. & LEES, G.M. Investigation of occurrence of tolerance to bronchodilator drugs in chronically pretreated guinea-pigs, 331

ANLEZARK, G.M., HORTON, R.W., MELDRUM, B.S., SAWAYA, M.C.B. & STEPHENSON, J.D. γ-Aminobutyric acid metabolism and the anticonvulsant action of ethanolamine-o-sulphate and di-n-propylacetate, 383P

ANLEZARK, G.M., MARSDEN, C.D. & MELDRUM, B.S. A primate model of acute dystonic reaction to neuroleptics, 381P

ASHFORD, J. & JONES, B.J. The effects of intra-amygdaloid injections of 6-hydroxydopamine on avoidance responding in rats, 255

ASSEM, E.S.K. Inhibition by antigen and by histamine antagonists of the uptake of histamine by isolated human leucocytes, 347P

B

BAKER, DOROTHEA J, TRIST, D.G. & WEATHERALL, M Inhibition of phagocytosis by mepacrine, 346P

BAKER, J B.E. The episcope for large screen projection of tracings on opaque media during their recording for demonstrations, 396P

BAKHLE, Y.S. The nature of the bradykinin inactivating system in isolated lungs, 349P

BAKHLE, Y.S. & YOUDIM, M.B.H. Metabolism of phenylethylamine in rat isolated perfused lung: evidence for monoamine oxidase type B in lung, 125

BARASI, S, PARRY, O & ROBERTS, M.H T. The interpretation of responses of motoneurone field potentials to 5-hydroxytryptamine, 366P

BARBER, H.E., CALVEY, T.N, MUIR, K.T. & TAYLOR, K. Effect of edrophonium on erythrocyte acetylcholinesterase and neuromuscular function in the rat, 93

BERDE, B *see* RICHARDSON, B.P., 356P

BILSKI, A. & WALE, J. (introduced by DAWES, P.M.) Two types of intrinsic sympathomimetic activity with β-adrenoceptor blocking drugs, 362P

BLACK, J.W. *see* WAN, BEATRICE, Y.C., 357P

BLACKWELL, C.J. & FLOWER, R.J. Effects of steroid hormones on tissue levels of prostaglandin 15-hydroxydehydrogenase in the rat, 343P

BLAKELEY, A.G H. & SUMMERS, R.J. The effects of AH 5158 on the overflow of transmitter and the uptake of $[^3H]$-(-)-noradrenaline in the cat spleen, 364P

BOSIN, T.R, HIXSON, E. JANE & MAICKEL, R.P. Pressor effects of tryptamine analogues, 25

BOWERY, N.G. & JONES, G.P. A comparison of γ-aminobutryric acid and the semi-rigid analogues 4-aminotetrolic acid, 4-aminocrotonic acid and imidazole-4-acetic acid on the isolated superior cervical ganglion of the rat, 323

BRADLEY, N.J & POULTER, L.W (introduced by LEWIS, G.P.) *In vitro* methods of assessing the physiological activation of macrophages *in vivo*, 387P

BRANDTZAEG, P, GAUTVIK, K.M., NUSTAD, K. & PIERCE, J.V. Rat submandibular gland kallikreins: purification and cellular localization, 155

BROWN, D.A. & KWIATKOWSKI, D. A note on effect of dithiothreitol on depolarization of isolated sympathetic ganglia by carbachol and bromo-acetylcholine, 128

BROWN, D.A. *see* GARTHWAITE, J., 353P

BROWNLEE, G. *see* ABU-MURAD, C., 377P

BROWNLEE, G. *see* LITTLETON, J.M., 370P

BUCKINGHAM, J.C. & HODGES, J.R. Hypothalamo-pituitary adrenocortical function in the rat after treatment with betamethasone, 235

BUCKINGHAM, R.E., HAMILTON, T.C. & MOORE, R.A. Prolonged effects of *p*-chlorophenylalanine on blood pressure of conscious normotensive and DOCA/saline hypertensive rats, 69

BUNTING, S., MONCADA, S., NEEDLEMAN, P. & VANE, J.R Formation of prostaglandin endoperoxides and rabbit aorta contracting substance (RCS) by coupling two enzyme systems, 344P

BUSHMAN, J.A, ENDERBY, D.H., FOWLER, K.T. & PAYNE, J.P. The use of a mass spectrometer for the analysis and measurement of trace concentrations of anaesthetic vapours, 389P

BUTTAR, H.S. Impairment in the hepatic clearance of $[^{35}S]$-bromosulphophthalein in paracetamol-intoxicated rats, 145

C

CALVEY, T N *see* BARBER, H.E., 93

CANDY, J M. & KEY, B J. Blockade of the electrocortical changes induced by perfusion of amphetamine in the brain stem reticular formation, 374P

CARRIER, O., JR. & HESTER, R.K The relationship between calcium and increased sensitivity of rabbit aortae four hours after reserpine, 449

CARRUBA, M.O, GROPPETTI, A., MANTEGAZZA, P., VICENTINI, L & ZAMBOTTI, F. Effects of mazindol, a non-phenylethylamine anorexigenic agent on biogenic amine levels and turnover rate, 431

CHAHL, LORIS A. & LADD, R.J. The effects of prostaglandins E_1, E_2 and $F_{2\alpha}$ on the cutaneous vasculature of the rat, 317

CHAIT, L.D. *see* ADAMS, M.D., 43

CHANG, J , LEWIS, G.P. & PIPER, PRISCILLA J. The effects of anti-inflammatory steroids on levels of prostaglandin in adipose tissue *in vitro*, 324P

CHAPPLE, D J., ENDERBY, D H., HUGHES, R. & PAYNE, J P Neuromuscular and cardiovascular studies with dimethyl tubocurarine in anaesthetized cats, rhesus monkeys and man, 354P

CHIPMAN, P. & GLOVER, W.E. Histamine H_2-receptors in the human peripheral circulation, 494

CLARKE, D.E & ROMANYSHYN, W.M. Blood pressure responses to adrenal field stimulation as a measure of adrenal catecholamine release, 271

COLLIER, H.O.J. & FRANCIS, D.L Stereospecific suppression by opiates of the quasi-morphine abstinence syndrome elicited by 3-isobutyl-1-methylxanthine (IBMX), 382P

COLLIER, P.S. & SHANKS, R.G. Alterations to vasodilator effects of isoprenaline in the dog following intra-arterial infusions with isoprenaline, salbutamol and orciprenaline, 363P

CURTIS, D R., GAME, C.J.A. & LODGE, D Benzodiazepines and central glycine receptors, 307

CURZON, G & MARSDEN, C.A. Effect of LSD on rat brain 5-hydroxytryptamine metabolism at elevated environmental temperature, 368P

CUTHBERT, N.J., DINNEEN, L.C., FRANCIS, D.L & SCHNEIDER, C A rapid *in vivo* test for dependence potential of analgesic drugs, 386P

D

DANILOV, A.F., IVANOV, YU.J. & MICHELSON, M.J. Influence of motor nerve tetanization on effect of organophosphorus cholinesterase inhibitors on neuromuscular transmission, 115

DARGIE, H.J., FRANKLIN, S.S. & REID, J.L. The sympathetic nervous system and renovascular hypertension in the rat, 365P

DAWES, P.M *see* BILSKI, A., 362P

DEVOY, P.W. *see* ALPS, B.J., 179

DICK, W.C., GRENNAN, D.M. & ZEITLIN, I.J. Studies on the relative effects of prostaglandins, bradykinin, 5-hydroxytryptamine and histamine on the synovial microcirculation in dogs, 313

DINNEEN, L C. *see* CUTHBERT, N.J., 386P

DOLPHIN, A.C., ELLIOTT, P.N.C. & JENNER, P. Reduction of locomotor activity in mice by dopamine-β-hydroxylase inhibitors—evidence against the involvement of non-specific irritation, 395P

DONALDSON, I.M., JENNER, P., MARSDEN, C D., MILLER, R. & PERINGER, E. Is metoclopramide a directly acting dopamine receptor antagonist? 373P

DORRIS, R.L. & SHORE, P.A On the mechanism of action of clozapine on the adrenergic neurone, 279

DOW, R C. & LASZLO, I. Uptake of 5-hydroxytryptamine in different parts of the brain of the rabbit after intraventricular injection, 443

DRUMMOND, A H. & GORDON, J.L Uptake of 5-hydroxytryptamine by rat blood platelets and its inhibition by adenosine 5′-phosphate, 417

DUNCAN, A.J & WILSON, W.S. Some preservatives in eyedrop preparations hasten the formation of dryspots in the rabbit cornea, 359P

DUTTA, S., MARKS, B.H. & STEPHEN, P.M. The uptake and subcellular distribution of radio-labelled metabolites of digitoxin in the guinea-pig isolated perfused heart, 437

E

EARNHARDT, J T. *see* ADAMS, M.D., 43

EDWARDS, J.H., EVANS, ELIZABETH, EVANS, P.H., NICHOLLS, P.J. & RAJAN, K.T. Effect of a purified cotton dust extract on human lung in culture, 394P

ENDERBY, D.H., HUGHES, R., PAYNE, J.P & WORSLEY, R. Assessment of neuromuscular blockade in man by use of tetanic and single twitch contractions of the adductor pollicis muscle, 390P

ENDERBY, D H. *see* BUSHMAN, J.A., 389P

ENDERBY, D.H. *see* CHAPPLE, D.J., 354P

ELLIOTT, P.N.C. *see* DOLPHIN, A.C., 395P

EVANS, ELIZABETH, *see* EDWARDS, J.H., 394P

EVANS, JANE P.M., GRAHAME-SMITH, D.G., GREEN, A.R. & TORDOFF, ANN F.C. Electroconvulsive shock increases the behavioural responses of rats to brain 5-hydroxytryptamine accumulation and central nervous system stimulant drugs, 193

EVANS, P.H *see* EDWARDS, J.H., 394P

F

FARRINGTON, HELEN E. & OWEN, D.A A Measurement of vascular changes in acute inflammatory responses, 388P

FELTZ, ANNE & LARGE, W.A. Effect of atropine on decay of miniature end-plate potentials at frog neuromuscular junction, 111

FERREIRA, S.H., HERMAN, A.G. & VANE, J.R. Prostaglandin production by rabbit isolated jejunum and its relationship to the inherent tone of the preparation, 469

FILE, SANDRA E (introduced by NEAL, M.J.) Are central cholinergic pathways involved in the habituation of exploration and distraction? 378P

FINCH, L. & HERSOM, A. Studies on the centrally mediated cardiovascular effects of apomorphine in the anaesthetized rat, 366P

FLOWER, R.J., HARVEY, E.A. & KINGSTON, W.P. Inflammatory effects of prostaglandin D_2 in rat and human skin, 229

FLOWER, R J *see* BLACKWELL, C.J., 343P

FORD-HUTCHINSON, A.W., SMITH, M.J.H & WALKER, J.R. Chemotactic activity of solutions of prostaglandin E_1, 345P
FOWLER, K.T. *see* BUSHMAN, J.A., 389P
FRANCIS, D.L. *see* COLLIER, H.O. J., 382P
FRANCIS, D.L. *see* CUTHBERT, N.J., 386P
FRANKLIN, S.S *see* DARGIE, H.J , 365P
FRY, J.P., SHARMAN, D.F. & STEPHENS, D.B. The ontogenesis of cerebral dopamine metabolism in the pig, 372P
FRY, J.P., SHARMAN, D.F. & STEPHENS, D B. The effect of apomorphine on oral behaviour in piglets, 388P

G

GAME, C.J.A. *see* CURTIS, D.R., 307
GANGULY, D.K Antioxotremorine action of propranolol, 21
GARTHWAITE, J. (introduced by BROWN, D.A.) The uptake of weak acids and bases into isolated rat superior cervical ganglia in relation to intracellular pH, 353P
GAUTVIK, K.M. *see* BRANDTZAEG, P , 155
GILBERT, J C. & WYLLIE, M.G. Effects of anticonvulsant and convulsant drugs on the ATPase activities of synaptosomes and their components, 49
GLOVER, W E *see* CHIPMAN, P., 494
GOODLET, I , MIREYLEES, S.E & SUGRUE, M.F. The selective inhibition of 5-hydroxytryptamine re-uptake by Org 6582, 367P
GORDON, J.L. *see* DRUMMOND, A.H., 417
GRAHAME-SMITH, D G. *see* EVANS, JANE P.M , 193
GRANT, L. & POWIS, G. The effect of inhibitors of alcohol metabolism on the changes in the hepatic microsomal metabolism of foreign compounds produced by a single dose of ethanol in the rat, 376P
GRAY, J.A., LÜLLMANN, H., MITCHELSON, F. & REIL, G.-H Stereoselective binding in cardiac tissue of the enantiomers of benzetimide, an antimuscarinic drug, 485
GREEN, A.R. *see* EVANS, JANE P.M., 193
GRENNAN, D.M *see* DICK, W.C., 313
GRIFFITHS, P J. *see* ABU-MURAD, C., 377P
GROPPETTI, A. *see* CARRUBA, M.O., 431
GRYGLEWSKI, R.J. & KORBUT, R. Bioassay of histamine in the presence of prostaglandins, 39
GUTMAN, Y. & MAZUR-RUDER, M. Rat uterus renin-like activity: effect of stimuli and hormones, 285

H

HAEUSLER, G. & LEWIS, P.J. Sympathetic nerve recording in the conscious rabbit, 386P
HAMILTON, T.C. *see* BUCKINGHAM, R.E., 69
HARVEY, E.A. *see* FLOWER, R.J., 229
HERMAN, A.G. *see* FERREIRA, S.H., 469
HERSOM, A. *see* FINCH, L., 366P
HESTER, R.K. *see* CARRIER, O., JR., 449
HILL, R.G., SIMMONDS, M.A. & STRAUGHAN, D.W. Antagonism of γ-aminobutyric acid and glycine by convulsants in the cuneate nucleus of cat, 9
HILLIDGE, C.J. & LEES, P. Influence of etorphine, acepromazine and diprenorphine on cardiovascular function in ponies, 375P
HIMORI, N. & TAIRA, N. A method for recording smooth muscle and vascular responses of the blood-perfused dog trachea *in situ*, 293
HIROM, P C., MILLBURN, P & PARKER, R.J. (introduced by WILLIAMS, R.T.) The enterohepatic circulation of $[^3H]$-phenolphthalein in the rat, 355P
HIXSON, E. JANE *see* BOSIN, T.R , 25
HODGES, J.R. *see* BUCKINGHAM, J C., 235
HOLMES, S.W. & SUGDEN, D. The effect of melatonin on pinealectomy-induced hypertension in the rat, 360P
HOLZBAUER, MARGARETHE. Physiological aspects of the hypnotic properties of steroid hormones, 382P
HORTON, R.W. *see* ANLEZARK, G M., 383P
HUGHES, R. *see* CHAPPLE, D J., 354P
HUGHES, R. *see* ENDERBY, D.H., 390P
HUTCHINGS, R.T., LEWIS, G.P., SABIKOWSKI, Z.T. & WESTWICK, J. Measurement of changes in the microvasculature, 391P

I

IAVANOV, YU J *see* DANILOV, A.F., 115

J

JATON, A.L., LOEW, D.M. & VIGOURET, J.M. CF 25-397 (9,10-Didehydro-6-methyl-8β-[2-pyridylthiomethyl]-ergoline), a new central dopamine receptor agonist, 371P
JENKINSON, D.H. *see* KOLLER, K., 362P
JENNER, P. *see* DOLPHIN, A.C., 395P
JENNER, P. *see* DONALDSON, I.M., 373P
JOHNSON, A.M., LOEW, D.M. & VIGOURET, J M. Stimulant properties of bromocriptine on central dopamine receptors in comparison to apomorphine, (+)-amphetamine and L-DOPA, 59
JOHNSON, F.N. Effect of lithium chloride on one-trial passive avoidance learning in rats, 87
JONES, B.J. *see* ASHFORD, J., 255
JONES, G.P. *see* BOWERY, N.G., 323
JONES, R.L. Selectivity of action of some C15-modified prostaglandins D, 339P
JURKIEWICZ, A. & JURKIEWICZ, N H. Dural effects of α-adrenoceptor antagonists in rat isolated vas deferens, 169
JURKIEWICZ, N.H. *see* JURKIEWICZ, A., 169

K

KAROUM, F., MAJCHROWICZ, E. & WYATT, R. Brain concentrations of biogenic amine metabolites in acutely treated and ethanol-depleted rats, 403
KEY, B.J. *see* CANDY, J.M., 374P
KILLICK, S.W. *see* AMBACHE, N., 351P
KINGSTON, W.P. *see* FLOWER, R.J., 229
KOLLER, K. (introduced by JENKINSON, D.H.) An interaction between the α and β actions of catecholamines in guinea-pig liver slices, 362P
KORBUT, R. *see* GRYGLEWSKI, R.J., 39
KOSS, M.C. & NAKANO, J. Reflex bradycardia and hypotension produced by prostaglandin $F_{2\alpha}$ in the cat, 245

KUMAR, A., SHARMA, H L & SHARMA, V.N. Effect of SAS (a new 10-*N*-acylaminophenothiazine) on gastric secretion and ulceration in rats, 491
KWIATKOWSKI, D. *see* BROWN, D.A., 128

L

LADD, R.J *see* CHAHL, LORIS A., 317
LARGE, W.A. *see* FELTZ, ANNE, 111
LASZLO, I *see* DOW, R.C , 443
LEES, P. & SERRANO, L Effects of azaperone on cardiovascular and respiratory functions in the horse, 263
LEES, G.M. *see* ANDERSON, A.A., 331
LEES, P *see* HILLIDGE, C.J , 375P
LEWIS, A.J The assessment of systemically and topically administered anti-inflammatory drugs using u.v. erythema production in the rat, 385P
LEWIS, G P, PECK, M J, WILLIAMS, T J & YOUNG, BEVERLEY A., Measurements of blood flow in rabbit skin homografts and autografts using a ^{133}Xe-clearance technique, 396P
LEWIS, G.P *see* BRADLEY, N.J , 387P
LEWIS, G P *see* CHANG, J., 342P
LEWIS, G P *see* HUTCHINGS, R.T , 391P
LEWIS, G P. *see* PECK, M.J , 396P
LEWIS, G.P. *see* WILLIAMS, T.J., 341P
LEWIS, P.J. *see* HAEUSLER, G , 386P
LITTLETON, J M. & MACLEAN, K.I. (introduced by BROWNLEE, G.) Alterations in dopamine uptake in rat corpus striatum induced by combinations of stress and Δ^8-tetrahydrocannabinol (Δ^8-THC), 370P
LITTLETON, J M *see* ABU-MURAD, C., 377P
LODGE, D. *see* CURTIS, D R., 307
LOEW, D.M. *see* JATON, A.L., 371P
LOEW, D.M. *see* JOHNSON, A M , 59
LÜLLMAN, H. *see* GRAY, J.A., 485

M

McCALL, ELAINE & YOULTEN, L J.F Prostaglandin production by rat polymorphonuclear leucocytes, 396P
McGRATH, J.C & MacKENZIE, J.E. A pithed rabbit preparation for stimulation of different segments of the autonomic outflow, 395P
MacKENZIE, J.E. *see* McGRATH, J.C., 395P
MACLEAN, K.I. *see* LITTLETON, J.M., 370P
MAGOS, L. The effects of dimercaptosuccinic acid on the excretion and distribution of mercury in rats and mice treated with mercuric chloride and methylmercury chloride, 479
MAICKEL, R.P. *see* BOSIN, T R., 25
MAJCHROWICZ, E. *see* KAROUM, F., 403
MANTEGAZZA, P. *see* CARRUBA, M.O., 431
MANTOVANI, P. *see* AMADUCCI, L , 379P
MARCHAND, C. & NADEAU, D. The distribution and kinetics of sulphacetamide in leukaemic mice, 423
MARKS, B H. *see* DUTTA, S., 437
MARLEY, E. & WHELAN, JENNIFER E. Some pharmacological effects of *p*-chlorophenylalanine unrelated to tryptophan hydroxylase inhibition, 133
MARSDEN, C.A. *see* CURZON, G., 368P
MARSDEN, C D. *see* ANLEZARK, G.M., 381P
MARSDEN, C D. *see* DONALDSON, I.M., 373P
MAZUR-RUDER, M *see* GUTMAN, Y , 285
MELDRUM, B.S. *see* ANLEZARK, G.M., 381P
MELDRUM, B S. *see* ANLEZARK, G.M., 383P
MELROSE, JOANNE, PALFREYMAN, M.G., POYSER, R. H & WHITING, R L BRL 13776: a novel antihypertensive agent with interesting noradrenaline-depleting properties, 361P
MICHAL, F. & MOTAMED, MINA Shape change and aggregation of blood platelets: interaction between the effects of adenosine diphosphate, 5-hydroxytryptamine and adrenaline, 209
MICHELSON, M J. *see* DANILOV, A.F., 115
MICHELSON, M.J & SHELKOVNIKOV, S.A. Isotonic and isometric responses of different tonic muscles to agonists and antagonists, 457
MILLBURN, P. *see* HIROM, P.C., 355P
MILLER, R. *see* DONALDSON, I.M., 373P
MILSON, J.A & PYCOCK, C J Effects of drugs acting on cerebral 5-hydroxytryptamine mechanisms on dopamine-dependent turning behaviour in mice, 77
MIREYLEES, S.E. *see* GOODLET, I , 367P
MITCHELSON, F *see* GRAY, J.A , 485
MONCADA, S. *see* BUNTING, S., 344P
MOORE, R A *see* BUCKINGHAM, R.E., 69
MORTON, I.K.M., ROSE, G.E., SAVERYMUTTU, S.H. & WOOD, J.R. Effects of some fatty acids on gallbladder function, 350P
MOTAMED, MINA *see* MICHAL, F , 209
MUIR, K.T. *see* BARBER, H.E., 93

N

NADEAU, D. *see* MARCHAND, C., 423
NAHORSKI, S.R., PATTON, WENDY & ROGERS, K.J Developmental changes in the sensitivity of neurohormone-stimulated cyclic AMP formation in chick cerebral hemispheres, 380P
NAKANO, J. *see* KOSS, M.C., 245
NEAL, M.J. *see* FILE, SANDRA E., 378P
NEEDLEMAN, P. *see* BUNTING, S., 344P
NICHOLLS, P.J *see* EDWARDS, J.H., 394P
NIEDERHAUSER, U. & WHITTLE, B.J.R. A simple perfusion system for the study of histamine release from rat peritoneal mast cells, 391P
NISTRI, A. Effects of morphine on acetylcholine release from the frog spinal cord, 352P
NUSTAD, K. *see* BRANDTZAEG, P., 155

O

OSBORNE, N.N. & PENTREATH, V W. Effects of 5,7-dihydroxytryptamine on an identified 5-hydroxytryptamine-containing neurone in the central system of the snail *Helix pomatia*, 29
OWEN, D.A.A. *see* FARRINGTON, HELEN E , 388P

P

PALFREYMAN, M.G. *see* MELROSE, JOANNE, 361P
PARKER, R.J. *see* HIROM, P.C., 355P

PARRATT, J R. *see* ABAITEY, A.K , 219
PARRY, O. *see* BARASI, S., 366P
PATTON, WENDY *see* NAHORSKI, S R., 380P
PAYNE, J P *see* BUSHMAN, J A., 389P
PAYNE, J.P. *see* CHAPPLE, D.J , 354P
PAYNE, J.P *see* ENDERBY, D H , 390P
PECK, M.J & WILLIAMS, T.J. (introduced by LEWIS, G P.) Measurement of inflammatory exudation and local blood flow changes in rabbit skin, using ^{131}I-albumin and ^{133}Xe, 396P
PECK, M.J *see* LEWIS, G.P., 396P
PENTREATH, V.W. *see* OSBORNE, N.N., 29
PEPEU, G *see* AMADUCCI, L , 379P
PERINGER, E *see* DONALDSON, I.M., 373P
PIERCE, J.V. *see* BRANDTZAEG, P., 155
PIPER, PRISCILLA J. *see* CHANG, J., 342P
POULTER, L.W. *see* BRADLEY, N.J., 387P
POWIS, G. *see* GRANT, L., 376P
POYSER, R.H. *see* MELROSE, JOANNE, 361P
PYCOCK, C.J. *see* MILSON, J.A., 77

R

RAJAN, K T. *see* EDWARDS, J.H , 394P
REID, J L *see* DARGIE, H.J., 365P
REIL, G -H. *see* GRAY, J.A., 485
RICHARDSON, B.P. (introduced by BERDE, B.) The effects of cyproheptadine pretreatment on insulin release from isolated pancreatic islets, 356P
RICHARDSON, P.D I. The effects of intravenous secretin on the small intestinal vasculature of the cat, 358P
RIVERA-CALIMLIN, LEONOR Impaired absorption of chlorpromazine in rats given trihexyphenidyl, 301
ROBERTS, M.H T. *see* BARASI, S., 366P
ROGERS, K J. *see* NAHORSKI, S R., 380P
ROMANYSHYN, W.M. *see* CLARKE, D.E., 271
ROSE, G E. *see* MORTON, I.K.M., 350P

S

SABIKOWSKI, Z.T. *see* HUTCHINGS, R.T., 391P
SAVERYMUTTU, S.H. *see* MORTON, I.K.M., 350P
SAWAYA, M.C B. *see* ANLEZARK, G M., 383P
SAXENA, P.N. Sodium and calcium ions in the control of temperature set-point in the pigeon, 187
SCHILD, H O. Dale and the development of pharmacology, 3
SCHNEIDER, C. *see* CUTHBERT, N.J., 386P
SEIDLER, F.J. & SLOTKIN, T.A. Effects of chronic nicotine administration on the denervated rat adrenal medulla, 201
SERRANO, L. *see* LEES, P., 263
SHANKS, R.G. *see* COLLIER, P S., 363P
SHARMA, H.L. *see* KUMAR, A., 491
SHARMA, V.N. *see* KUMAR, A , 491
SHARMAN, D.F. *see* FRY, J.P., 372P
SHARMAN, D.F. *see* FRY, J.P., 388P
SHELKOVNIKOV, S.A. *see* MICHELSON, M.J., 457
SHORE, P.A *see* DORRIS, R.L., 279
SIMMONDS, M.A. *see* HILL, R.G., 9
SLOTKIN, T.A *see* SEIDLER, F.J., 201
SMITH, D.F. Lithium orotate, carbonate and chloride: pharmacokinetics, polydipsia and polyuria in rats, 399
SMITH, M.J H *see* FORD-HUTCHINSON, A.W., 345P
STEPHEN, P.M *see* DUTTA, S., 437
STEPHENS, D.B *see* FRY, J P., 372P
STEPHENS, D.B *see* FRY, J P , 388P
STEPHENSON, J.D *see* ANLEZARK, G.M , 383P
STONE, T.W. Responses of neurones in cerebral cortex and caudate nucleus to amantadine, amphetamine and dopamine, 101
STRAUGHAN, D.W. *see* HILL, R.G., 9
SUGDEN, D *see* HOLMES, S.W., 360P
SUGRUE, M.F *see* GOODLET, I., 367P
SUMMERS, R.J. *see* BLAKELEY, A.G.H., 364P

T

TAIRA, N. *see* HIMORI, N., 293
TAYLOR, K. *see* BARBER, H.E., 93
TORDOFF, ANN F C. *see* EVANS, JANE P.M., 193
TRIST, D.G. *see* BAKER, DOROTHEA, J., 346P

V

VANE, J R. *see* BUNTING, S , 344P
VANE, J.R *see* FERREIRA, S.H., 469
VERNEY, J. *see* ADEBANJO, A.O., 392P
VICENTINI, L. *see* CARRUBA, M.O., 431
VIGOURET, J.M. *see* JATON, A.L , 371P
VIGOURET, J.M. *see* JOHNSON, A.M., 59

W

WALE, J. *see* BILSKI, A., 362P
WALKER, J R *see* FORD-HUTCHINSON, A W., 345P
WAN, BEATRICE Y C. (introduced by BLACK, J.W.) Effects of dibutyryl cyclic AMP and phosphodiesterase inhibitors on acid secretion by mouse stomach *in vitro*, 357P
WATERFALL, J.F. *see* ALPS, B.J., 179
WEATHERALL, M. *see* BAKER, DOROTHEA M., 346P
WESTWICK, J. *see* HUTCHINGS, R T., 391P
WHELAN, JENNIFER E. *see* MARLEY, E., 133
WHITING, R.L. *see* MELROSE, JOANNE, 361P
WHITTLE, B.J.R. Prostaglandins and changes in the gastric mucosal barrier and blood flow during indomethacin- and bile salt-induced mucosal damage, 340P
WHITTLE, B J.R. *see* NIEDERHAUSER, U., 391P
WILLIAMS, R.T. *see* HIROM, P.C., 355P
WILLIAMS, T.J. (introduced by LEWIS, G.P.) The pro-inflammatory activity of E-, A-, D-, and F-type prostaglandins and analogues 16,16-dimethyl-PGE_2 and (15S)-15-methyl PGE_2 in rabbit skin; the relationship between potentiation of plasma exudation and local blood flow changes, 341P
WILLIAMS, T.J. *see* LEWIS, G.P., 396P
WILLIAMS, T J. *see* PECK, M.J., 396P
WILSON, W.S. *see* DUNCAN, A.J., 359P
WOOD, J.R. *see* MORTON, I.K.M., 350P
WORSLEY, R. *see* ENDERBY, R., 390P
WYATT, R *see* KAROUM, F., 403
WYLLIE, M.G. *see* GILBERT, J.C., 49

Y

YOUDIM, M.B.H. Monoamine oxidase active site; the binding to and titration of monoamine oxidase with [^{14}C]-selective inhibitors, 375P
YOUDIM, M.B.H. *see* BAKHLE, Y.S., 125
YOULTEN, L.J.F. *see* McCALL, ELAINE, 396P
YOUNG, BEVERLEY A *see* LEWIS, G.P., 396P

Z

ZAMBOTTI, F. *see* CARRUBA, M.O., 431
ZEITLIN, I.J *see* DICK, W.C., 313

SUBJECT INDEX

A

Acepromazine, influence of etorphine with, and antagonistic effects of diprenorphine on cardiovascular function in ponies, 375P
Acetaldehyde oxime, effect of, on liver microsomal metabolism of compounds produced by single dose of ethanol in rats, 376P
Acetylcholine, causes dose-dependent increases in blood flow rate and tracheal constriction in dog, 293
—, effects of α-adrenoceptor antagonists on responses of rat isolated vas deferens to, 169
—, effect of, on hepatic microsomal metabolism, 376P
—, effects of, on isotonic and isometric responses of different muscles, 457
—, effect of, on transmission to internal anal sphincter, 392P
—, effect of morphine on release of, from frog spinal cord, 352P
—, effect of some phospholipids on output of, from cerebral cortex in the rat, 379P
—, has no effect on prostaglandin release by contracting isolated rabbit jejunum, 469
—, increased sensitivity to, of rabbit aortic strips following reserpine, 449
—, spasmolytic effect of SAS in response to, in rats, 491
—, use of, to detect changes in activity of synaptic cholinesterases in cat muscle, 115
Acetylcholinesterase, in rat erythrocytes, effect of edrophonium on, 93
Acute dystonic reaction, a primate model of, to neuroleptics, 381P
ACTH$_{1\text{-}24}$, incubation of adipose tissue with, causes only small amounts of prostaglandins to be released, 342P
Adductor pollicis muscle, assessment of neuromuscular blockade in man by use of tetanic and single twitch contractions of, 390P
Adenosine, cyclic AMP responsiveness to, in chick cerebral hemispheres, 380P
Adenosine diphosphate, interaction of effects of, and 5-HT on blood platelets, 209
—, potentiates histamine- and prostaglandin-induced inflammatory responses in rabbit skin, 341P
—, shape change and aggregation of blood platelets induced by, enhanced by simultaneous addition of 5-HT, 209
Adenosine 5′-phosphate, inhibition of uptake of 5-HT by rat blood platelets, 417
Adenosine triphosphate, -ase, effects of anticonvulsant and convulsant drugs on, activities of synaptosomes and their components, 49
Adenylate cyclase, activity of, in chick cerebral hemispheres, 380P
Adipose tissue, effects of anti-inflammatory steroids on prostaglandin levels in, *in vitro*, 342P
Adrenalectomy, effect of, on tissue levels of prostaglandin 15-hydroxydehydrogenase in the rat, 343P
—, has no effect on uterine renin-like activity in the rat, 285
Adrenaline, causes vasoconstriction in dog trachea, 293
—, effect of, on blood pressure response of adrenal medulla, 271
—, has no effect on prostaglandin release by relaxing isolated rabbit jejunum, 469
—, interaction between effects of ADP, 5-HT and, on shape change and aggregation of blood platelets, 209
—, isotonic contractile responses of isolated rat vas deferens to different doses of, effects of yohimbine, piperoxan, phentolamine and tolazoline on, 169
—, pretreatment with, may cause development of tolerance to relaxant effects of, in guinea-pig trachea, 331
—, relaxes tracheal smooth muscle in dog, 293
α-Adrenoceptors, present in dog tracheal musculature and vasculature, 293
α-Adrenoceptor antagonists, dual effect of, in rat isolated vas deferens, 169
α-Adrenoceptor blocker, acepromazine has some action as an, 375P
β-Adrenoceptors, present in dog tracheal musculature and vasculature, 293
β-Adrenoceptor blockers, two types of intrinsic sympathomimetic activity with, 362P
Adrenocorticotrophic hormone, circulating levels of, following betamethasone treatment in the rat, 235
AH 5158, effects of, on the overflow of transmitter and uptake of [^{3}H]-(-)noradrenaline in cat spleen, 364P
—, 5-(1-Hydroxy-2-((1-methyl-3-phenylpropyl)amino) ethyl)salicylamide, 364P
Alcohol, effect of inhibitors of metabolism of, on changes in hepatic microsomal metabolism of foreign compounds produced by single dose of ethanol in the rat, 376P
Amantadine, response of cerebral cortex and caudate nucleus neurones to, 101
Amberlite XAD-2, column used in bioassay of histamine, 39
Amidephrine, causes hyperpolarization and potentiation in guinea-pig liver slices, 362P
4-Aminocrotonic acid, effect of, on isolated superior cervical ganglion of the rat, 323
—, reduces ganglionic accumulation of [^{3}H]-GABA, 323
2-Amino-6-methyl-5-oxo-4-*n*-propyl-4,5-dihydro-*s*-triazolo (1,5-a)pyrimidine, see ICI 63197, 375P
4-Aminotetrolic acid, effect of, on isolated superior cervical ganglion of the rat, 323
—, has no effect on ganglionic accumulation of [^{3}H]-GABA, 323
(+)-Amphetamine, effect of, on central dopamine receptors, 59
Amphetamine, blockade of electrocortical changes induced by perfusion of, in brain stem reticular formation, 374P

—, effect of, on turning behaviour in mice, 77
—, mimics electrocortical changes induced by noradrenaline without secondary sedative effects, 374P
—, responses of neurones in cerebral cortex and caudate nucleus to, 101
2-*n*-Amylthio-AMP, abolishes ADP-induced inhibition of uptake of 5-HT by rat platelets, 417
Anaesthetics, use of mass spectrometer for analysis and measurement of trace concentrations of vapours of, 389P
Analgesics, a rapid *in vivo* test for dependence potential of, 386P
Angiotensin II, reduces exudation potentiation in histamine- and prostaglandin-induced inflammation in rabbit skin, 341P
Anterior hypothalamus, uptake of 5-HT by, following intraventricular injection, 443
Anticonvulsant drugs, effects of, on ATPase activities of synaptosomes and their components, 49
Antigen, inhibits uptake of histamine by isolated human leucocytes, 347P
Anti-IgE, inhibits uptake of histamine by isolated human leucocytes, 347P
Anti-inflammatory drugs, assessment of systemically and topically administered, using ultraviolet erythema production in the rat, 385P
Aortic spiral preparation, guinea-pig, Wy 20051 contracts isolated, 179
Apomorphine, effect of, on central dopamine receptors, 59
—, effect of, on oral behaviour in piglets, 388P
—, effect of, on turning behaviour in mice, 77
—, studies on the centrally mediated cardiovascular effects of, in the anaesthetized rat, 366P
Arachidonic acid, effects of, on gallbladder function, 350P
—, has no effect on prostaglandin release by isolated rabbit jejunum, but contracts the preparation, 469
Aspirin, anti-inflammatory efficacy of, administered topically and systemically, 385P
Asthma, use of diethylcarbamazine citrate in treatment of, 219
Atropine, antagonizes acetylcholine-induced muscle and vascular responses in dog trachea, 293
—, effect of, on amine uptake in rat heart, 279
—, effect of, on decay of miniature end-plate currents at frog neuromuscular junction, 111
—, effect of, on motor response of pig retractor penis muscle, 351P
—, effect of, on responses of cat pulmonary system to $PGF_{2\alpha}$, 245
—, pretreatment with, abolishes cardiovascular effects of apomorphine in anaesthetized rat, 366P
[^{3}H]-Atropine, uptake of, by isolated rat superior cervical ganglia, 353P
Atropine sulphate, effect of, on habituation of exploration and distraction in rats, 378P
Avoidance responses, effects of intra-amygdaloid injections of 6-OHDA on, in rats, 255
Azaperone, effects of, on cardiovascular and respiratory function in the horse, 263

B

Barium chloride, spasmolytic activity of SAS in response to, in rats, 491
Benzalkonium chloride, hastens formation of dryspots in rabbit corneas, 359P
Benzetimide, stereoselective winding in cardiac tissue of enantiomers of, an antimuscarinic drug, 485
Benzhexol hydrochloride, effect of, on habituation of exploration and distraction in rats, 378P
Benzodiazepines, and central glycine receptors, 307
Benzotropine, abolishes dystonic reaction in response to chlorpromazine in baboons, 381P
Betamethasone, effect of, on prostaglandin production in adipose tissue, 342P
—, hypothalamo-pituitary adrenocortical function in rat following treatment with, 235
—**-17-valerate,** anti-inflammatory efficacy of, administered topically and systemically, 385P
Bethanidine, effect of, on cardiovascular responses of cat to diethylcarbamazine citrate, 219
Bicuculline, blocks responses to GABA and three of its analogues of isolated superior cervical ganglion of the rat, 323
(+)-Bicuculline, methochloride, antagonizes GABA and glycine in cat cuneate nucleus, 9
Bile salt, gastric mucosal damage induced by, and effect of prostaglandins on, 340P
Blood flow, measurements of changes in, and inflammatory exudation using ^{131}I-albumin and ^{133}Xe, 396P
—, measurement of, in rabbit skin homografts and autografts using a ^{133}Xe- clearance technique, 396P
Blood platelets, shape change and aggregation of, interaction between effects of ADP, 5-HT and adrenaline, 209
Blood pressure, effects of SAS on, in anaesthetized rats, 491
—, prolonged effects of *p*-chlorophenylalanine on, in conscious normotensive and DOCA/saline hypertensive rats, 69
Bradykinin, addition of PGE_1 or PGE_2 to, before intradermal injection potentiates plasma exudation, 341P
—, has vasodilator effect on dog synovial circulation, 313
—, increased vascular permeability in rat skin induced by, unaffected by PGD_2, 229
—, relative effects of prostaglandins, 5-HT and histamine on synovial microcirculation in dogs, 313
—, the nature of the, -inactivating system in isolated lungs, 349P
Bretylium, effect of, on motor response of pig retractor penis muscle, 351P
—, effect of, on transmission to internal anal sphincter, 392P
BRL 13776, a novel antihypertensive agent with interesting noradrenaline-depleting properties, 361P
Bromo-acetylcholine, depolarization of isolated sympathetic ganglia by, effect of DTT on, 128
Bromocriptine, stimulant properties of, on central dopamine receptors in comparison to apomorphine, (+)-amphetamine and L-DOPA, 59
[^{35}S]-Bromosulphophthalein, hepatic clearance of, impaired in paracetamol-intoxicated rats, 145
Bronchodilators, investigation of occurrence of tolerance to, in chronically pretreated guinea-pigs, 331
Butoxamine, effect of, on transmission to internal anal sphincter, 392P

C

Caffeine, comparative effects of, and triazolopyrimidine and theophylline on acid secretion in mouse stomach, 357P

Calcium, effect of, in Ringer solution in measuring acetylcholine output from rat cerebral cortex, 379P
—, has no effect on renin-like activity in rat uterus, 285
—, ions in the control of temperature set-point in the pigeon, 187
—, relationship between, and supersensitivity of rabbit aortae following reserpine, 449
—, uptake but not metabolism of, in isolated pancreatic islets affected by cyproheptadine, 356P
Calcium chloride, contractile response of rabbit aortic strips to, potentiated by reserpine, 449
Carbachol, effects of, isotonic and isometric responses of different muscles, 457
—, depolarization of isolated sympathetic ganglia by, effect of DTT on, 128
—, use of, to detect changes in activity of synaptic cholinesterases in cat muscle, 115
Carrageenan-induced oedema, potentiated by PGD_2, 229
Catecholamine, depletion of, in and round amygdalae caused by intra-amygdaloid injections of 6-OHDA, 255
—, metabolism of, and liver dysfunction during induction of ethanol dependence, 377P
—, metabolites of, concentrations in brain increased by ethanol treatment, 403
—, nicotine treatment of intact rat adrenal glands causes reversible depletion of, 201
—, not decreased in denervated rat adrenal medulla following chronic nicotine administration, 201
—, renal release of, measured by blood pressure responses to adrenal field stimulation, 271
—, an interaction between the α and β actions of, in guinea-pig liver slices, 362P
Caudate nucleus, neurones in, responses of, to amantadine, amphetamine, and dopamine, 101
Central cholinergic pathways, are they involved in habituation of exploration and distraction? 378P
Cerebral cortex, neurones in, responses of, to amantadine, amphetamine and dopamine, 101
Cerebrospinal fluid, relative sodium and calcium ion concentrations in, has important function in temperature control in birds, 187
CF 25-397, 9,10-didehydro-6-methyl-8β[2-pyridylthiomethyl]ergoline, a new central dopamine receptor agonist, 371P
Chlordiazepoxide, effects of, on spinal neurones and antagonism between strychnine and glycine, 307
Chlorhexidine, hastens formation of dryspots in rabbit corneas, 359P
Chlorimipramine, compared with Org 6582 as an inhibitor of re-uptake of 5-HT, 367P
Chlorisondamine, effect of, on amine uptake in rat heart, 279
—, effect of, on blood pressure response to field stimulation of adrenal gland and intravenous adrenaline, 271
***p*-Chloroamphetamine**, ability of, to lower rat brain 5-HT levels as method for studying blockade of 5-HT re-uptake, 367P
***dl*-8-Chloro-11-anti-amino-benzo-(b)-bicyclo-[3.3.1]nona-3,6a(10a)diene hydrochloride**, see Org 6582, 367P
2-Chloro-10-[4'(*N*-β-hydroxyethyl) piperazinyl-1'] acetylphenothiazine, see SAS, 491
***p*-Chlorophenylalanine**, pretreatment with, has no effect on depressor response to apomorphine of anaesthetized rat, 366P
—, prolonged effects of, on blood pressure of conscious normotensive and DOCA/saline hypertensive rats, 69
—, some pharmacological effects of, unrelated to tryptophan hydroxylase inhibition, 133
Chlorpromazine, dystonic response to, in baboon, 381P
—, effect of, on amine uptake in rat heart, 279
—, effect of, on responses of neurones of cerebral cortex and caudate nucleus to amantadine and dopamine, 101
—, impaired absorption of, in rats given trihexyphenidyl, 301
—, metabolism of, by liver homogenates not affected by treatment with trihexyphenidyl, 301
—, plasma and brain levels of, lowered after oral administration of trihexyphenidyl, 301
Cholinesterase, organophosphorus inhibitors, influence of motor nerve tetanization on effect of, on neuromuscular transmission, 115
Circling behaviour, in mice, effects of drugs acting on 5-HT mechanisms and dopamine-dependent, 77
Clomipramine, effect of, on turning behaviour in mice, 77
Clozapine, effects of, on amine uptake in rat brain, 279
—, may have incomplete reserpine-like effect on adrenergic neurone facilitating amine release in rat heart, 279
—, mechanism of action of, on adrenergic neurone, 279
Cocaine, effects of piperoxan and yohimbine on responses of rat isolated vas deferens to, 169
Codeine, suppression of QMAS by, 386P
Compound 48/80, effect of, on prostaglandin-induced changes in rat cutaneous vasculature, 317
Convulsant drugs, effects of, on ATPase activities of synaptosomes and their components, 49
Cornea, rabbit, some preservatives in eyedrop preparations hasten formation of dryspots in, 359P
Cortex, uptake of 5-HT into various areas of, following intraventricular injection, 443
Corticosteroids, have no effect on ACTH-induced stimulation of glycerol levels in adipose tissue, 342P
—, inhibit release of, but not synthesis of, prostaglandins in adipose tissue, 342P
Corticosterone, levels of, following betamethasone treatment in rat, 235
Cotton dust extract, purified, effect of, on human lung in culture, 394P
Cuneate nucleus, antagonism of GABA and glycine by convulsants in cat, 9
Cyclic AMP, developmental changes in sensitivity of neurohormone-stimulated formation of, in chick cerebral hemispheres, 380P
—, responses of guinea-pig liver slices to, potentiated by α-adrenoceptor agonists, 362P
Cyproheptadine, effect of, on turning behaviour in mice, 77
—, effects of pretreatment with, on insulin release from isolated pancreatic islets, 356P

D

Dale, Sir Henry, and the development of pharmacology, Centennial Symposium, 3
Deoxycorticosterone acetate, effect of, on prolonged effects of *p*-chlorophenylalanine on blood pressure in conscious normotensive and DOCA/saline hypertensive rats, 69
—, administration of, has no effect on uterine renin-like activity in the rat, 285
Desipramine, compared with Org 6582 as an inhibitor of re-uptake of 5-HT, 367P

—, effect of, on blood pressure response to field stimulation of adrenal gland and intravenous adrenaline, 271

—, lowers heart amine concentrations in rat, 279

Desmethylimipramine, has no effect on uptake of tritiated Δ^1-tetrahydrocannabinol by isolated vas deferens, 413

—, inhibits uptake of tritiated noradrenaline in isolated rat vas deferens, 413

Dexamethasone, anti-inflammatory efficacy of, administered topically and systemically, 385P

—, effect of, on tissue levels of prostaglandin 15-hydroxy-dehydrogenase in the rat, 343P

Dexetimide, antagonistic potency, uptake and washout of, compared with levetimide, in guinea-pig cardiac tissue, 485

Dextrorphan, has no effect on QMAS, 382P

Diazepam, effect of, on ATPase activities of synaptosomes, 49

—, effect of, on potency of strychnine as a convulsant, 307

—, intravenously in cats, effect of, on dorsal root potentials, 307

Dibutyryl cyclic AMP, effects of, on acid secretion by mouse stomach *in vitro,* 357P

—, responses of guinea-pig liver slices to, potentiated by α-adrenoceptor agonists, 362P

Dichlorisoprenaline, produces dose-dependent tachycardia in rats pretreated with syrosingopine, 362P

9,10-Didehydro-6-methyl-8β[2-pyridylthiomethyl]ergoline, see CF 25-398, 371P

Diethycarbamazine citrate, cardiovascular effects of, 219

Digitoxigen-bis-digitoxoside, uptake and inotropic effects of, in the isolated perfused guinea-pig heart, 437

Digitoxigenin-mono-digitoxoside, uptake and inotropic effects of, in the isolated perfused guinea-pig heart, 437

Digitoxigenin, uptake and inotropic effects of, in the isolated perfused guinea-pig heart, 437

Digitoxin, uptake and subcellular distribution of radio-labelled metabolites of, in guinea-pig isolated perfused heart, 437

3,4-Dihydroxypenylacetic acid, brain concentrations of, increased in rats by ethanol treatment, 403

—, in pig brain during first few weeks of life, 372P

—, levels of, in metoclopramide-treated mice, 373P

5,6-Dihydroxytryptamine, more potent than 5,7-DHT at blocking uptake of 5-HT into snail CNS neurone, 29

5,7-Dihydroxytryptamine, blocks uptake of $[^3H]$-5-HT into neurone, 29

—, decreases endogenous 5-HT content of snail CNS neurone, 29

—, effects of, on an identified 5-HT-containing neurone in the snail central nervous system, 29

—, neurotoxic for indoleamine-containing neurones, 29

—, produced post-synaptic blockade of transmission by 5-HT blocking, 29

Dimercaptosuccinic acid, effects of, on excretion and distribution of mercury in rats and mice treated with mercuric chloride and methylmercury chloride, 479

—, four times more efficient than D-penicillamine in reducing body burden of mercury, 479

$[^{14}C]$-Dimethyl-2,4-oxazolidinedione, uptake of, by isolated rat superior cervical ganglia, 353P

Dimethyltubocurarine, effect of, on contractions of pig retractor penis muscle, 351P

—, neuromuscular and cardiovascular studies with, in anaesthetized cats, rhesus monkeys and man, 354P

Dinitrophenol, has no effect on prostaglandin release by relaxing isolated rabbit jejunum, 469

Diprenorphine, influence of etorphine acepromazine and, on cardiovascular function in ponies, 375P

Di-n-propylacetate, GABA metabolism and anticonvulsant action of, 383P

Disulphiram, effect of, on hepatic microsomal metabolism, 376P

Dithiothreitol, effect of, on depolarization of isolated sympathetic ganglia by carbachol and bromo-acetylcholine, 128

Dodecamethonium, effects of, on isotonic and isometric responses of different muscles, 457

L-DOPA, effect of, on central dopamine receptors, 59

—, locomotor activity following administration of, enhanced by repeated electroconvulsive shocks, 193

Dopamine, alterations in uptake of, in rat corpus striatum induced by combinations of stress and Δ^8-tetrahydro-cannabinol, 370P

—, brain concentrations of, following administration of mazindol, 431

—, brain concentrations of metabolites of, in acutely-treated and ethanol-dependent rats, 403

—, central receptors, stimulant properties of bromocriptine on, in comparison to apomorphine, (+)-amphetamine and L-DOPA, 59

—, CF 25-397, a new central, receptor agonist, 371P

—, -dependent turning behaviour in mice, effects of drugs acting on cerebral 5-HT mechanisms on, 77

—, effect of 6-OHDA on brain concentrations of, 255

—, ontogenesis of cerebral metabolism of, in the pig, 372P

—, rat brain levels of, unaltered by Org 6582, 367P

—, receptor agonist, is metoclopramide one? 373P

—, responses of neurones in cerebral cortex and caudate nucleus to, 101

—, striatal concentrations of, unaffected by intra-amygdaloid injections of 6-OHDA, 255

—, synthesis and release of, following metoclopramide treatment, 373P

—, turnover in brain enhanced by mazindol following blockade by α-methyl-p-tyrososine, 431

Dopamine β-hydroxylase, increases in both intact and denervated rat adrenal glands during chronic nicotine administration, 201

Dopamine-β-hydroxylase inhibitors, reduction of locomotor activity in mice by, as evidence against the involvement of non-specific irritation, 395P

Dopamine receptor mechanism, striatal, circling behaviour due to, in mice, 77

Dopaminergic nerve terminals, nigrostriatal effects of drugs acting on cerebral 5-HT mechanisms on dopamine-dependent turning behaviour in mice with unilateral destruction of, 77

Dorsal muscle, leech, responses of, to different agonists and antagonists, 457

E

Edrophonium, effect of, on erythrocyte acetylcholinesterase and neuromuscular function in rat, 93

Electroconvulsive shock, increases behavioural responses of rats to brain 5-HT accumulation and CNS stimulant drugs, 193

Electroconvulsive therapy, therapeutic effect of, discussed relative to effects of repeated electroconvulsive shocks on behavioural responses, 193
Episcope, for large screen projection of tracings on opaque media during their recording for demonstrations, 396P
Erythema, long-lasting, dose-related, produced by intradermal injection of PGD$_1$ and PGD$_2$ in human forearm, 229
Erythrocytes, rat, acetylcholinesterase in, effect of edrophonium on, 93
Ethanol, catecholamine metabolism and liver dysfunction during induction of dependence on, 377P
—, effects of acute and chronic treatment with, on brain concentrations of biogenic amine metabolites in rats, 403
—, single dose of, effect of inhibitors of alcohol metabolism on changes in hepatic microsomal metabolism of foreign compounds produced by, 376P
Ethanolamine-o-sulphate, GABA metabolism and anticonvulsant action of, 383P
Ethosuximide, effect of, on ATPase activities of synaptosomes, 49
Etorphine, influence of, with acepromazine and antagonistic effects of diprenorphine on cardiovascular function in ponies, 375P

F

933F, 2-Piperidinomethyl-1,4-benzodioxan hydrochloride, effects of, on overflow of transmitter and uptake of noradrenaline in cat spleen, 364P
Flufenamic acid, anti-inflammatory efficacy of, administered topically and systemically, 385P
Fluoxetine, compared with Org 6582 as an inhibitor of re-uptake of 5-HT, 367P
Fluphenazine, pretreatment with, abolishes depressor response to apomorphine in anaesthetized rat, 366P

G

GABA, antagonism of, and glycine by convulsants in cat cuneate nucleus, 9
—, comparison of, and three semi-rigid analogues on isolated superior cervical ganglion of the rat, 323
—, metabolism of, and anticonvulsant action of ethanolamine-o-sulphate and di-n-propylacetate, 383P
Gall bladder, effects of some fatty acids on function of, 350P
Ganglia, isolated sympathetic, depolarization of, by carbachol and bromoacetylcholine, effect of DTT on, 128
Gastric mucosal barrier, changes in, and blood flow during induced mucosal damage, 340P
Glucose, insulin release induced by, abolished by pretreatment with cyproheptadine, in rat pancreatic islets, 356P
β-Glucuronidase, release of, as in indication of the effects of cotton dust on human lung tissue, 394P
Glutamate, effect of, on motoneurone field potentials, 366P
Glycerol, increased levels of, during ACTH stimulation of adipose tissue, 342P
Glycine, antagonism of GABA and, by convulsants in cat cuneate nucleus, 9
—, benzodiazepines and mammalian central receptors for, 307
—, effect of, on motoneurone field potentials, 366P
Guanethidine, effect of, on blood pressure response of adrenal medulla, 271
—, effect of, on motor response of pig retractor penis muscle, 351P
—, effect of, on transmission to the internal anal sphincter, 392P

H

Haloperidol, dystonic response to, in baboon, 381P
—, effect of, on amine uptake in rat heart, 279
—, pretreatment with, abolishes depressor response to apomorphine of anaesthetized rat, 366P
7 HBK, inactivation of, in isolated rat lungs, 349P
8 HBK, inactivation of, in isolated rat lungs, 349P
Helix pomatia, effects of 5,7-dihydroxytryptamine on a 5-HT-containing neurone of, central nervous system, 29
Hepatic triglycerides, accumulation of, during induction of ethanol dependence, 377P
Heptamethonium, effects of, on isotonic and isometric responses of different muscles, 457
Heroin, overcomes QMAS in dose-related way, 382P
Hexamethonium, effect of, on blood pressure response to field stimulation of adrenal gland and intravenous adrenaline, 271
—, effect of, on cardiovascular responses to diethylcarbamazine citrate in cat, 219
—, effect of pretreatment with, on response to intravenous secretin in the cat small intestine, 358P
Histamine, addition of PGE$_1$ or PGE$_2$ to, before intradermal injection potentiates plasma exudation, 341P
—, bioassay of, in presence of prostaglandins, 39
—, cyclic AMP responsiveness to, in chick cerebral hemispheres, 380P
—, effect of ICI 63197 on acid secretory responses of mouse stomach to, 357P
—, increased vascular permeability in rat skin induced by, potentiated by PGD$_2$, 229
—, infused into brachial artery causes increase in forearm blood flow, 494
—, inhibition by antigen and antagonists of, of the uptake of, by isolated human leucocytes, 347P
—, relative effects of prostaglandins, 5-HT, bradykinin and, on synovial microcirculation in dogs, 313
—, simple perfusion system for study of release of, from rat peritoneal mast cells, 391P
—, spasmolytic effect of SAS in response to, in rats, 491
—, has vasodilator effect on dog synovial circulation, 313
Histamine H$_2$-receptors, in human peripheral circulation, 494
8-β Homo Phe-bradykinin, see 8 HBK, 349P
7-β Homo Pro-bradykinin, see 7 HBK, 349P
Homovanillic acid, brain concentrations of, increased in rats by ethanol treatment, 403
—, in pig brain during first few weeks of life, 372P
—, levels of, in metoclopramide-treated mice, 373P
Hydrocortisone, anti-inflammatory efficacy of, administered topically and systemically, 385P
—, effect of, on prostaglandin production in adipose tissue, 342P
Hydrocortisone succinate, effect of, on tissue levels of prostaglandin 15-hydroxydehydrogenase, 343P

5-Hydroxy-5-*p*-chlorophenyl-2,3-dihydro-5H-imidazo-2,1-a-isoindole, see mazindol, 431

6-Hydroxydopamine, effects of intra-amygdaloid injections of, on avoidance response in rats, 255

—, effects of, on amphetamine- or noradrenaline-induced electrocortical changes in cat brain stem reticular formation, 374P

—, effects of, on blood pressure and plasma noradrenaline in renovascular hypertension in the rat, 365P

—, lesions, CF 25-397 induces dose-dependent contralateral turning in rats with, 371P

—, pretreatment with, inhibits uptake of tritiated noradrenaline in isolated rat vas deferens, 413

—, reduces uptake of tritiated Δ^1-tetrahydrocannabinol by isolated vas deferens, 413

5-Hydroxyindole acetic acid, levels of, in LSD- and heat-treated rats, 368P

—, pargyline-induced decline of, unmodified by administration of mazindol, 431

—, rat brain levels of, decreased by Org 6582, 367P

—, single electroconvulsive shock increases concentration of rat brain, 193

4-(2-Hydroxy-3-isopropylaminopropoxy)phenyl-acetamide, see ICI 66082, 362P

***p*-Hydroxymandelic acid,** brain concentrations of, in rats unaffected by ethanol treatment, 403

5-(1-Hydroxy-2-((1-methyl-3-phenylpropyl)amino)ethyl) salicylamide, see AH 5158, 364P

***p*-Hydroxyphenylacetic acid,** brain concentrations of, in rats unaffected by ethanol treatment, 403

11β-Hydroxy-16α,17α,21-trimethyl-prena-1,4-diene-3,20-dione, see Org 6216, 385P

5-Hydroxytryptamine, brain concentrations of following administration of mazindol, 431

—, comparison of effects of, with PCPA on various tissue preparations from different species, 133

—, effects of drugs acting on cerebral, mechanisms on dopamine-dependent turning behaviour in mice, 77

—, effect of 6-OHDA on brain concentrations of, 255

—, effect of single electroconvulsive shock on synthesis of, 193

—, enhances effects of ADP on blood platelets, 209

—, effect of LSD on rat brain metabolism of, at elevated environmental temperature, 368P

—, has vasodilator effect on dog synovial circulation, 313

—, interaction of effects of, and ADP on blood platelets, 209

—, interpretation of responses of motoneurone field potentials to, 366P

—, relative effects of prostaglandins, bradykinin, and histamine on synovial microcirculation in dogs, 313

—, selective inhibition of re-uptake of, by Org 6582, 367P

—, single electroconvulsive shock has no effect on rat brain concentrations of, 193

—, uptake of, by rat blood platelets and its inhibition by adenosine 5′-phosphate, 417

—, uptake of, in different parts of the rabbit brain after intraventricular injection, 443

5-Hydroxytryptophan, effect of, on cerebral 5-HT mechanisms and dopamine-dependent turning behaviour in mice, 77

Hyoscine, abolishes dystonic reaction in response to chlorpromazine in baboons, 381P

—, has no effect on prostaglandin release by relaxing isolated rabbit jejunum, 469

Hyperalgesia, potentiated by large doses of PGD$_2$, 229

Hypertension, blood pressure during, prolonged effects of *p*-chlorophenylalanine on, in rats, 69

—, induced by pinealectomy, partly caused by lack of normal melatonin secretion, 360P

Hypotension, induced in horses as a result of azaperone injection, 263

Hypovolaemia, has no effect on uterine renin-like activity in the rat, 285

I

ICI 63197, 2-Amino-6-methyl-5-oxo-4-*n*-propyl-4,5-dihydro-*s*-triazolo(1,5-a)pyrimidine, 357P

—, effects of, on acid secretory responses to histamine and pentagastrin, 357P

ICI 66082, 4-(2-hydroxy-3-isopropylaminopropoxy)phenyl-acetamide, comparison of haemodynamic effects of, in dogs with those of practolol and pindolol, 362P

Ileum muscles, guinea-pig, responses of, to different agonists and antagonists, 457

Imidazole-4-acetic acid, effect of, on isolated superior cervical ganglion of the rat, 323

—, reduces ganglionic accumulation of [^{3}H]-GABA, 323

Imipramine, has no effect on ADP or 5-HT effects on blood platelets, 209

Indomethacin, antagonizes effects of arachidonic acid, but has little effect on those of ricinoleic acid, in gallbladder function, 350P

—, anti-inflammatory efficacy of, administered topically and systemically, 385P

—, decreases prostaglandin production by rabbit isolated jejunum and relaxes the preparation, 469

—, effect of, on gastric mucosa, 340P

—, gastric mucosal damage induced by, and effect of prostaglandins on, 340P

—, has no effect on ACTH-induced stimulation of glycerol levels in adipose tissue, 342P

—, reduces total amount of prostaglandin formed during lipolysis in adipose tissue, 342P

Inflammatory exudation, measurement of, and local blood flow changes in rabbit skin, using ^{131}I-albumin and ^{133}Xe, 396P

Inflammatory responses, measurement of vascular changes in acute, 388P

Insulin, release of, from isolated pancreatic islets following pretreatment with cyproheptadine, 356P

Internal anal sphincter, inhibitory transmission to, 392P

3-Isobutyl-1-methylxanthine, stereospecific suppression by opiates of the quasi-morphine abstinence syndrome elicited by, 382P

Isometric response, of different tonic muscles to agonists and antagonists, 457

Isoprenaline, alterations to vasodilator effects of, in the dog following intra-arterial infusions with isoprenaline, salbutamol and orciprenaline, 363P

—, causes vasodilatation in dog trachea, 293

—, cyclic AMP responsiveness to, in chick cerebral hemispheres, 380P

—, increased sensitivity to, of rabbit aortic strips following reserpine, 449

—, injection of, has no effect on uterine renin-like activity in the rat, 285

—, relaxes tracheal smooth muscle in dog, 293

—, response of guinea-pig liver slices to, potentiated by α-receptor agonists, 362P
—, tolerance to relaxant effects of, not affected by pretreatment with salbutamol, 331
Isotonic response, of tonic muscles to different agonists and antagonists, 457

J

Jejunum, prostaglandin production by rabbit isolated, and inherent tone of the preparation, 469

K

Kallikreins, rat submandibular gland, purification and cellular localization of, 155
Krebs medium, incubation of adipose tissue in, causes release of small amount of PGE_2, 342P
—, calcium-free, has no effect on prostaglandin release by relaxing isolated rabbit jejunum, 469
—, containing indomethacin, effect of, on prostaglandin release by isolated rabbit jejunum after storage in, 469

L

Learning, one-trial passive avoidance, in rats, effect of lithium chloride on, 87
Lecture given at Sir Henry Dale Centennial Symposium, Cambridge, 17–19 September 1975, 3
Levetimide, antagonistic potency, uptake and washout of, compared with dexetimide, in guinea-pig cardiac tissue, 485
Levorphanol, suppresses QMAS, 382P
Lithium, comparison of pharmacokinetics of ionic, administered as the orotate, carbonate or chloride, 399
Lithium carbonate, pharmacokinetics of, polydipsia and polyuria in rats, 399
Lithium chloride, effect of, on one-trial passive avoidance learning in rats, 87
—, pharmacokinetics of, polydipsia and polyuria in rats, 399
Lithium orotate, pharmacokinetics of, polydipsia and polyuria in rats, 399
LSD, effect of, on rat brain 5-HT metabolism at elevated environmental temperature, 368P
—, effect of, on transmission to internal anal sphincter, 392P
—, effect of, on turning behaviour in mice, 77
Lung, rat isolated perfused, metabolism of phenylethylamine in, evidence for MAO type B in lung, 125
Lymphokine, effect of, on macrophage activation *in vivo*, 387P

M

Macrophages, *in vitro* methods of assessing the physiological activation of, *in vivo*, 387P
Magnesium, effect of, instead of calcium, in Ringer solution in measuring acetylcholine output from rat cerebral cortex, 379P
—, ions, effects of, perfused into lateral cerebral ventricle of pigeon, 187
Mass spectrometer, use of, for analysis and measurement of trace concentrations of anaesthetic vapours, 389P
Mast cells, simple perfusion system for study of histamine release from rat peritoneal, 391P
Mazindol, a non-phenylethylamine anorexigenic agent, 431
—, effects of, on biogenic amine levels and turnover rate, 431
Medulla oblongata, uptake of 5-HT into, following intraventricular injection, 443
Melatonin, effect of, on pinealectomy-induced hypertension in the rat, 360P
Mepacrine, inhibition of phagocytosis by, 346P
Mepyramine, effect of, on motor response of pig retractor penis muscle, 351P
—, inhibits uptake of histamine by isolated human leucocytes, 347P
—, reduces histamine-induced increase in forearm blood flow, 494
Mercuric chloride, effects of dimercaptosuccinic acid on excretion and distribution of mercury in rats and mice treated with, 479
Mesenteric vasculature preparation, rat perfused, Wy 20051 evokes constrictor responses in, 179
Metaraminol, clozapine lowers heart concentrations of, 279
(-)-Metaraminol, -induced fall in rat heart noradrenaline levels unaffected by Org 6582, 367P
Methionine, cell content of, decreased by 5,7-dihydroxytryptamine, 29
Methoxamine, causes hyperpolarization and potentiation in guinea-pig liver slices, 362P
—, pretreatment with, or adrenaline has no effect on sensitivity to contractor action of methoxamine, in guinea-pig trachea, 331
5-Methoxy *N,N*-dimethyltryptamine, hyperactivity following administration of, enhanced by repeated electroconvulsive shocks, 193
3-Methoxy-4-hydroxyphenylglycol, brain concentrations of, increased in rats by ethanol treatment, 403
Methylfurmethide, effects of, on isotonic and isometric responses of different muscles, 457
Methylmercury chloride, effects of dimercaptosuccinic acid on excretion and distribution of mercury in rats and mice treated with, 479
α-Methyl-*p*-tyrosine, effect of, on response of neurones of cerebral cortex and caudate nucleus to amantadine, amphetamine and dopamine, 101
—, effect of, on stimulant properties of bromocriptine on central dopamine receptors, 59
—, pretreatment with reserpine and, decreases and delays dystonic response to haloperidol in baboons, 381P
Methysergide, effect of, on prostaglandin-induced changes in rat cutaneous vasculature, 317
—, effect of, on turning behaviour in mice, 77
—, inhibits 5-HT effects on ADP-induced shape and aggregation changes of blood platelets, 209
—, prevents PCPA effects on rat fundus and cat adrenal medulla, 133
Metiamide, effect of, on effects of dibutyryl cyclic AMP on gastric acid secretion in the mouse, 357P
—, inhibits uptake of histamine by isolated human leucocytes, 347P
—, reduces histamine-induced increases in forearm blood flow, 494

Metoclopramide, a directly acting dopamine receptor agonist? 373P
—, pretreatment with, abolishes depressor response to apomorphine in anaesthetized rat, 366P
Microvasculature changes, apparatus for measurement for, 391P
Miniature end-plate currents, decay of, at frog neuromuscular junction, effect of atropine on, 111
Monoamine oxidase, binding to and titration of, with $[^{14}C]$-selective inhibitors to investigate an active MAO site, 375P
—, type B in lung, evidence for, 125
Morphine, effect of, on acetylcholine release from frog spinal cord, 352P
$[^{14}C]$-Morphine, uptake of, by isolated rat superior cervical ganglia, 353P
Muscarinic receptors, present in dog tracheal musculature and vasculature, 293

N

Naloxone, effects of, on acetylcholine release from frog spinal cord, 352P
—, reverses heroin-induced suppression of QMAS, 382P
Naphazoline, causes hyperpolarization and potentiation in guinea-pig liver slices, 362P
Nephrectomy, bilateral, has no effect on uterine renin-like activity in the rat, 285
Nerve, motor, tetanization of, influence of, on effect of organophosphorus cholinesterase inhibitors on neuromuscular transmission, 115
Neuroleptics, primate model of acute dystonic reaction to, 381P
Neuromuscular blockade, assessment of, in man by use of tetanic and single twitch contractions of adductor pollicis muscle, 390P
Neuromuscular function, in rat, effect of edrophonium on, 93
Neuromuscular junction, frog, decay of miniature end-plate potentials at, effect of atropine on, 111
Neuromuscular transmission, influence of motor nerve tetanization on effect of organophosphorus cholinesterase inhibitors on, 115
Neurones, in cerebral cortex and caudate nucleus, responses of, to amantadine, amphetamine and dopamine, 101
Nicotine, cardiovascular effects of diethylcarbamazine citrate similar to those induced by, 219
—, effects of chronic administration of, on denervated rat adrenal medulla, 201
—, effect of, on transmission to internal anal sphincter, 391P
$[^3H]$-Nicotine, uptake of, by isolated rat superior cervical ganglia, 353P
Nitrogen, gas bubbled through preparation of rabbit isolated jejunum, decreases prostaglandin release and relaxes preparation, 469
Noradrenaline, amphetamine mimics electrocortical changes induced by, 374P
—, as a measure of peripheral sympathetic activity in renovascular hypertension in the rat, 365P
—, brain concentrations of, following administration of mazindol, 431
—, brain concentrations of metabolites of, in acutely-treated and ethanol-dependent rats, 403
—, causes hyperpolarization and potentiation in guinea-pig liver slices, 362P
—, causes vasoconstriction in dog trachea, 293
—, clozapine lowers heart concentrations of, 279
—, effect of 6-OHDA on brain concentrations of, 255
—, effects of, on prostaglandin-induced changes in rat cutaneous vasculature, 317
—, effect of Org 6582 on re-uptake of, in rat heart, 367P
—, increased sensitivity to, of rabbit aortic strips following reserpine, 449
—, inhibits phenoxybenzamine-induced twitching of pig retractor penis muscle, 351P
—, intravenously, effect of, on cardiovascular effects of Δ^9-tetrahydrocannabinol, 43
—, isotonic contractile responses to different doses of, effects of yohimbine, piperoxan, phentolamine and tolazoline on, 169
—, levels depleted in various peripheral organs by administration of BRL 13776, 361P
—, pressor response curve of, shifted to right of control following dose of Wy 20051, 179
—, relaxes tracheal smooth muscle in dog, 293
—, released by diethylcarbamazine citrate, 219
$[^3H]$-(-)Noradrenaline, effects of AH 5158 on overflow of transmitter and uptake of, in cat spleen, 364P
$[^3H]$-Noradrenaline, uptake of, by isolated rat vas deferens, 413

O

Octopamine, brain concentrations of metabolites of, in acutely-treated and ethanol-dependent rats, 403
Oedema, rat paw test, not elicited by PGD_2, 229
Oestradiol-17β, effect of, on tissue levels of prostaglandin 15-hydroxydehydrogenase in the rat, 343P
Orciprenaline, alterations to vasodilator effects of isoprenaline in the dog following intra-arterial infusions with, 363P
Org 6216, 11β-hydroxy-$16\alpha,17\alpha$,21-trimethyl-pregna-1,4-diene-3,20-dione, anti-inflammatory efficacy of, administered topically and systemically, 385P
Org 6582, decreases rat brain 5-hydroxyindoleacetic acid levels, 367P
—, selective inhibition of 5-HT re-uptake by, 367P
Organophosphorus inhibitors, effect of, on neuromuscular transmission, effect of motor nerve tetanization on, 115
Ovariectomy, causes fall in uterine renin-like activity in the rat, 285
—, effect of, on tissue levels of prostaglandin 15-hydroxydehydrogenase in the rat, 343P
Oxoprenolol, produces dose-dependent tachycardia in rats pretreated with syrosingopine, 362P
Oxotremorine, influence of propranolol on neuromuscular and other effects of, 21
Oxygen, gas bubbled through preparation of rabbit isolated jejunum, effect on prostaglandin production and muscle tone, 469
Oxymetazoline, causes hyperpolarization and potentiation in guinea-pig liver slices, 362P

P

Papaverine, has no effect on prostaglandin release by relaxing isolated rabbit jejunum, 469

Paracetamol, causes maximal hepatic damage in rats following a time lag after acute administration, 145
—, hepatic uptake, metabolism and excretion of bromosulphophthalein reversibly impaired following liver injury caused by, 145
Pargyline, reduces brain concentrations of homovanillic acid and 3,4-dihydroxyphenylacetic acid in control and ethanol acutely-treated rats, 403
PCPA, *p*-chlorophenylalanine, 133
—, effects of, on various tissues from rat, cat, guinea-pig, 133
—, has a 5-HT-like action and can release a prostaglandin-like substance from guinea-pig lungs, 133
D-Penicillamine, decrease in body burden of mercury by, compared with that of dimercaptosuccinic acid, 479
Penicillin, effect of, on GABA and glycine in cat cuneate nucleus, 9
Pentagastrin, effect of ICI 63197 on acid secretory responses of mouse stomach to, 357P
Pentazocine, suppression of QMAS by, 386P
Pentobarbitone, cardiovascular effects of diethylcarbamazine citrate in cats anaesthetized with, 219
Pentolinium, effect of, on transmission to the internal anal sphincter, 392P
7-n-Pentyl-4-[1-(2-naphthylmethyl)-1,2,5,6-tetrahydro-4-pyridyl]-2,2-dimethylchroman-5-ol, see BRL 13776, 361P
Pethidine, suppression of QMAS by, 386P
pH, Intracellular, in relation to uptake of weak acids and bases into isolated rat superior cervical ganglia, 353P
Pharmacology, Dale and the development of, 3
Pharynx protractor muscle, holothurian, responses of, to different agonists and antagonists, 457
[^{14}C]-Phenobarbitone, uptake of, by isolated rat superior cervical ganglia, 353P
[^{3}H]-Phenolphthalein, enterohepatic circulation of, in the rat, 355P
Phenoxybenzamine, abolishes motor response of dog retractor penis muscle, 351P
—, effect of, on heart amine concentrations in the rat, 279
—, effects of, on prostaglandin-induced changes in rat cutaneous vasculature, 317
—, has no effect on phentolamine-induced reduction of contraction of pig retractor penis muscle, 351P
—, effect of, on transmission to internal anal sphincter, 392P
—, pretreatment with, reduced pressor effect of tryptamine and two of its analogues, 25
Phentolamine, abolishes α-mediated responses induced in guinea-pig liver slices, 362P
—, abolishes motor response of dog retractor penis muscle, 351P
—, blocks vasoconstriction response of dog trachea to adrenaline and noradrenaline, 293
—, effect of, on adrenaline- and noradrenaline-induced effects on rat isolated vas deferens, 169
—, effect of, on blood pressure response to field stimulation of adrenal gland and intravenous adrenaline, 271
—, effect of, on cardiovascular responses of cat to diethylcarbamazine citrate, 219
—, effect of, on heart amine concentrations in the rat, 279
—, effect of, on transmission to internal anal sphincter, 392P
—, slightly reduces motor response of pig retractor penis muscle, 351P
Phenylbutazone, anti-inflammatory efficacy of, administered topically and systemically, 385P
Phenylephrine, causes hyperpolarization and potentiation in guinea-pig liver slices, 362P
2-Phenylethanol, hastens formation of dryspots in rabbit corneas, 359P
Phenylethylamine, metabolism of, in rat isolated perfused lung: evidence for monoamine oxidase type B in lung, 125
Phenylmercuric nitrate, has no effect on rate of corneal drying in rabbits, 359P
Phenytoin, effect of, on ATPase activities of synaptosomes, 49
Phosphatidylcholine, effect of, on acetylcholine output from rat cerebral cortex, 379P
Phosphatidylethanolamine, effect of, on acetylcholine output from rat cerebral cortex, 379P
Phosphatidylserine, effect of, on acetylcholine output from rat cerebral cortex, 379P
Phosphodiesterase inhibitors, effects of, on acid secretion by mouse stomach *in vitro*, 357P
Phospholipids, effects of, on acetylcholine output from rat cerebral cortex, 379P
Physostigmine, has no effect on prostaglandin release by contracting isolated rabbit jejunum, 469
Picrotoxin, antagonizes GABA and glycine in cat cuneate nucleus, 9
Pimozide, blocks contralateral turning induced by CF 25-397 in dopamine lesioned rats, 371P
—, dystonic response to, in baboon, 381P
—, effect of, on stimulant properties of bromocriptine on central dopamine receptors, 59
Pindolol, produces dose-dependent tachycardia in rats pretreated with syrosingopine, 362P
γ-Piperidinobutyramide, see Wy 20051, DF480, 179
2-Piperidinomethyl-1,4-benzodioxan hydrochloride, see 933F, 364P
Piperoxan, effect of, on adrenaline- and noradrenaline-induced effects on rat isolated vas deferens, 169
—, pretreatment with, has no effect on cardiovascular response of anaesthetized rat to apomorphine, 366P
Pithed rabbit preparation, for stimulation of different segments of the autonomic outflow, 395P
Polydipsia, development of, in rats given lithium orotate, carbonate or chloride compared, 399
Polymorphonuclear leucocytes, prostaglandin production by rat, 396P
Polyuria, development of, in rats given lithium orotate, carbonate or chloride compared, 399
Potassium, effect of, on motoneurone field potentials, 366P
—, enhances renin-like activity in rat uterus, 285
—, entry of, into granulocytes reduced by mepacrine, 346P
—, increased levels of, in perfusion medium, increased uptake of digitoxin and metabolites in guinea-pig isolated perfused heart, 437
—, ions, effects of, perfused into lateral cerebral ventricle of pigeon, 187
—, reserpine decreases relaxation rate following contraction induced by, in rabbit aortic strips, 449
Potassium ions, change in ratio of sodium and, allows ADP-induced inhibition of 5-HT uptake by rat platelets, 417
Practolol, produces dose-dependent tachycardia in rats pretreated with syrosingopine, 362P
Pregnancy, effect of, on renin-like activity of uterus in the rat, 285

—, effect of, on tissue levels of prostaglandin 15-hydroxydehydrogenase in the rat, 343P

Pregnanolone, hypnotic effects of, 382P

[^{14}C]-Procaine, uptake of, by isolated rat superior cervical ganglia, 353P

Proceedings of the British Pharmacological Society, Royal College of Surgeons, 17th-19th December, 1975, 339P

Progesterone, effect of, on tissue levels of prostaglandin 15-hydroxydehydrogenase in the rat, 343P

—, effect of, on uterine renin-like activity in the rat, 285

***d*-Propoxyphene,** suppression of QMAS by, 386P

Propranolol, antioxotremorine action of, 21

—, blocks noradrenaline-induced inhibition of phenoxybenzamine-induced twitching of pig retractor penis muscle, 351P

—, blocks tracheal relaxation responses to isoprenaline, adrenaline and noradrenaline, 293

—, blocks vasodilatation induced by isoprenaline in dog trachea, 293

—, comparison of haemodynamic effects of, in dogs with those of practolol and pindolol, 362P

—, effect of, on amine uptake in rat heart, 279

—, effect of, on blood pressure response to field stimulation of adrenal gland and intravenous adrenaline, 271

—, effect of, on transmission to internal anal sphincter, 392P

—, effect of, on potentiated response of guinea-pig liver slices to isoprenaline, salbutamol and cyclic AMP, 362P

—, effect of pretreatment with, on response to intravenous secretin in the cat small intestine, 358P

—, has no effect on phentolamine-induced reduction of contraction of pig retractor penis muscle, 351P

—, inhibits neuromuscular blocking of oxotremorine in isolated rat phrenic nerve diaphragm preparation, 21

—, intensifies paralytic effect of tubocurarine in rat diaphragm, 21

—, prevents antagonism of tubocurarine paralysis by tetraethylammonium, 21

***n*-Propyl *p*-hydroxybenzoate,** hastens formation of dryspots in rabbit corneas, 359P

Prostaglandins, and changes in gastric mucosal barrier and blood flow during indomethacin- and bile salt-induced mucosal damage, 340P

—, bioassay of histamine in presence of, 39

—, effects of anti-inflammatory steroids on levels of, in adipose tissue, 342P

—, production of. by rabbit isolated jejunum and relationship to the inherent tone of the preparation, 469

—, production of, by rat polymorphonuclear leucocytes, 396P

—, pro-inflammatory activity of, and two analogues of PGE$_2$ in rabbit skin, 341P

—, relative effects of, bradykinin, 5-HT and histamine on synovial microcirculation in dogs, 313

Prostaglandin endoperoxides, formation of, and rabbit aorta contracting substance by coupling two enzyme systems, 344P

Prostaglandin 15-hydroxydehydrogenase, effects of steroid hormones on tissue levels of, in the rat, 343P

PGA$_1$, has less potentiating activity than PGEs in rabbit skin inflammation, 341P

PGA$_2$, has less potentiating activity than PGEs in rabbit skin inflammation, 341P

PGD, modified, show high pressor activity, 339P

—, selectivity of action of some C15-modified, 339P

PGD$_2$, inflammatory effects of, in rat and human skin, 229

—, only potentiates rabbit skin inflammatory exudates at high doses, 341P

PGE, comparison of effects of, in erythema production with those of PGD, 229

PGE$_1$, abolishes ADP-induced inhibition of uptake of 5-HT by rat platelets, 417

—, bioassay of histamine in presence of, 39

—, chemotactic activity of solutions of, 345P

—, effects of, on cutaneous vasculature of the rat, 317

—, has similar potentiating potency in rabbit skin inflammation as PGE$_2$, 341P

—, has vasodilator effect in dog synovial circulation, 313

—, sub-threshold doses of, do not potentiate vasodilator action of bradykinin in dog synovium, 313

PGE$_2$, administration of 15-methyl analogue of, reduces effects of combined taurocholate and indomethacin administration on gastric mucosa, 340P

—, bioassay of histamine in presence of, 39

—, comparison of effects of, and analogues of, with those of PGD$_2$ and PGF$_{2\alpha}$ analogues, 339P

—, continuously generated by isolated rabbit jejunum, effect of, on inherent tone of preparation, 469

—, effects of, on cutaneous vasculature of the rat, 317

—, had similar potentiating potency in rabbit skin inflammation as PGE$_1$, 341P

—, has vasodilator effect on dog synovial circulation, 313

PGF$_{1\alpha}$, has no vasodilatory effect on dog synovial circulation, 313

PGF$_{2\alpha}$, bioassay of histamine in the presence of, 39

—, comparison of effects of analogues of, with those of PGD$_2$ and PGE$_2$ analogues, 339P

—, effects of, on cutaneous vasculature of the rat, 317

—, has vasodilator effect on dog synovial circulation, 313

—, intravenously, causes dose-dependent increase in pulmonary arterial pressure, decrease in systemic arterial pressure and delayed bradycardia, 245

—, only potentiates rabbit skin inflammatory exudates at high doses, 341P

—, reflex bradycardia and hypotension produced by, in the cat, 245

—, release of, by rabbit isolated jejunum and inherent tone of the preparation, 469

—, tracheal pressure effects of, inhibited by diethylcarbamazine citrate, 219

PGG$_2$, conversion of, to thromboxane A$_2$ in blood, 344P

—, effects of, on rabbit aorta, 344P

PGH$_2$, effects of, on rabbit aorta, 344P

Pyrazole, effect of, on liver microsomal metabolism of compounds produced by single dose of ethanol in rats, 376P

Q

Quasi-morphine abstinence syndrome, stereospecific suppression by opiates of the, elicited by 3-isobutyl-1-methylxanthine, 382P

QMAS, quasi-morphine abstinence syndrome, 382P

R

Rabbit aorta contracting substance, formation of, and prostaglandin endoperoxides by coupling two enzyme systems, 344P

Rectus abdominus muscle, frog, responses of, to different agonists and antagonists, 457
Renin, kidney, uterine renin-like activity independent of, 285
—, rat uterus renin-like activity, effect of stimuli and hormones on, 285
—, uterine renin-like activity is hormone independent, 285
Renovascular hypertension, sympathetic nervous system and, in the rat, 365P
Reserpine, abolishes tyramine-induced pressor responses in rats, 179
—, amine release in rat heart induced by, delayed by ganglionic blockade, 279
—, CF 25-397 inhibits catalepsy induced by, 371P
—, decreases relaxation rate from a potassium-induced contraction of rabbit aortic strips, 449
—, effect of, on blood pressure responses of adrenal medulla to adrenaline, 271
—, effect of, on responses of neurones of cerebral cortex and caudate nucleus to amantadine, amphetamine and dopamine, 101
—, effect of, on stimulant properties of bromocriptine on central dopamine receptors, 59
—, increased calcium uptake and $^{45}Ca^{2+}$ efflux in rabbit aortic strips, 449
—, potentiates dose-response curve of pressor action of Wy 20051, 179
—, pretreatment with, abolishes tyramine-induced responses of pig retractor penis muscle, 351P
—, pretreatment with, and α-methyl-*p*-tyrosine, decreases and delays dystonic response to haloperidol in baboons, 381P
—, pretreatment with, reduced pressor effect of tryptamine, 25
—, treatment with, and subsequent relationship between calcium and supersensitivity of rabbit aorta, 449
Reticular formation, brain stem, blockade of electrocortical changed induced by perfusion of amphetamine in, 374P
Retractor dentis muscle, sea urchin, responses of, to different agonists and antagonists, 457
Retractor penis muscle, species variation in motor transmission to, 351P
Ricinoleic acid, effects of, on gallbladder function, 350P

S

Salbutamol, alterations to vasodilator effects of isoprenaline in the dog following intra-arterial infusions with, 363P
—, pretreatment with, prevents development of tolerance to relaxant actions of adrenaline, isoprenaline or salbutamol in guinea-pig trachea, 331
—, response of guinea-pig liver slices to, potentiated by α-receptor agonists, 362P
SAS, a new 10-*N*-acylaminophenothiazine, 491
—, effect of, on gastric secretion and ulceration in rats, 491
Scopolamine hydrobromide, effect of, on habituation of exploration and distraction in rats, 378P
Secretin, intravenously, effects of, on the small intestinal vasculature of the cat, 358P
Sodium, has no effect on renin-like activity in rat uterus, 285
—, ions in the control of temperature set-point in the pigeon, 187
Sodium chloride, effect of, on one-trial passive avoidance learning in rats, 87
Sodium ions, change in ratio of potassium and, allows ADP-induced inhibition of 5-HT uptake by rat platelets, 417
Sodium taurocholate, effects of, on gastric mucosa, 340P
[^{14}C]-Sorbitol, uptake of, by isolated rat vas deferens, 413
Steroid hormones, physiological aspects of hypnotic properties of, 382P
Stilboestrol, causes significant increase of uterine renin-like activity in the rat, 285
Stress, alterations in dopamine uptake in rat corpus striatum induced by combinations of, and Δ^8-THC, 370P
Strychnine, antagonizes GABA and glycine in cat cuneate nucleus, 9
—, effect of diazepam on potency of, as a convulsant in mice, 307
Substantia nigra, uptake of 5-HT by, following intraventricular injection, 443
Sulphacetamide, distribution and kinetics of, in leukaemic mice, 423
Superior cervical ganglia, uptake of weak acids and bases into isolated rat, in relation to intracellular pH, 353P
Sympathetic nerve recording, in the conscious rabbit, 386P
Synaptosomes, effects of anticonvulsant and convulsant drugs on ATPase activities of, 49

T

Tachycardia, induced in horses as a result of azaperone injection, 263
Testosterone, effect of, on uterine renin-like activity in the rat, 285
Tetanization, of motor nerve, influence of on effect of organophosphorus cholinesterase inhibitors on neuromuscular transmission, 115
Δ^1-Tetrahydrocannabinol, uptake of tritiated, by isolated rat vas deferens, 413
Δ^8-Tetrahydrocannabinol, alterations in dopamine uptake in rat corpus striatum induced by combinations of stress and, 370P
Δ^9-Tetrahydrocannabinol, intravenously, produces transient pressor response and then sustained hypotension and bradycardia in rat, 43
—, peripheral effects of, no tolerance to, 43
—, tolerance to the cardiovascular effects of, in the rat, 43
Tetrahydrozoline, causes hyperpolarization and potentiation in guinea-pig liver slices, 362P
Tetramethylenedisulphotetramine, blocks responses to GABA and three of its analogues of isolated superior cervical ganglion in the rat, 323
Tetrodotoxin, abolishes contractions of retractor penis muscle induced by electrical stimulation, 351P
—, effect of, on transmission to the internal anal sphincter, 392P
Theophylline, comparative effects of, and triazolopyrimidine and caffeine on acid secretion in mouse stomach, 357P
—, insulin release induced by, unaffected by pretreatment with cyproheptadine in rat pancreatic islets, 356P
Thiomersal, has no effect on rate of corneal drying in rabbit, 359P
Thioridazine, effect of, on amine uptake in rat heart, 279
Thromboxane A_2, conversion of prostaglandin G_2 to, is enzymic, 344P
Tolazoline, effect of, on adrenaline- and noradrenaline-induced effects on rat isolated vas deferens, 169

Tolbutamide, insulin release induced by, abolished by pretreatment with cyproheptadine, in rat pancreatic islets, 356P

Trachea, blood-perfused dog, method for recording smooth muscle and vascular response of, *in situ*, 293

Tramazoline, causes hyperpolarization and potentiation in guinea-pig liver slices, 362P

Tranylcypromaine, locomotor activity following administration of, enhanced by repeated electroconvulsive shocks, 193

Triazolopyrimidine, comparative effects of, and theophylline and caffeine on acid secretion in mouse stomach, 357P

Trihexyphenidyl, delays gastric emptying in rats as measured by clearance of a marker, 301

—, impairs absorption of chlorpromazine in rats, 301

[^{14}C]-Trimethylamine, uptake of, by isolated rat superior cervical ganglia, 353P

Tryptamine, pressor effects of analogues of, 25

Tryptophan, cell content of, decreased by 5,7-dihydroxytryptamine, 29

—, effects of, on 5-HT metabolism in LSD- and heat-treated rats, 368P

—, single electroconvulsive shock has no effect on rat brain concentrations of, 193

L-**Tryptophan,** effect of, on cerebral 5-HT mechanisms and on dopamine-dependent turning behaviour in mice, 77

Tryptophan hydroxylase, inhibition, some pharmacological effects of *p*-chlorophenylalanine unrelated to, 133

Tuberculin, -sensitized leucocytes show increased uptake of histamine, 347P

Tubocurarine, paralysis of rat diaphragm by, antagonized by propranolol, 21

(+)-**Tubocurarine,** antagonizes GABA and glycine in cat cuneate nucleus, 9

—, effects of, on isotonic and isometric responses of different muscles, 457

Turning behaviour, in mice, effects of drugs acting on cerebral 5-HT mechanisms on dopamine-dependent, 77

Tyramine, brain concentrations of metabolites of, in acutely-treated and ethanol-dependent rats, 403

Tyrosine, brain levels of, increase steadily during induction of ethanol dependence, 377P

[^{3}H]-Tyrosine, conversion index of, into brain noradrenaline decreased by mazindol, 431

Tyrosine hydroxylase, induced by chronic nicotine administration in intact and denervated rat adrenal glands, 201

U

Ultraviolet erythema, production of, in rats, in assessing systemically and topically administered anti-inflammatory drugs, 385P

V

Vanilmandelic acid, brain concentrations of, unaffected by acute ethanol treatment, but increased by chronic treatment, 403

Vas deferens, dual effect of α-adrenoceptor antagonists in rat isolated, 169

Vasodilatation, in rabbit skin may be responsible for potentiation of inflammatory exudation produced by prostaglandins, 341P

W

Wy 20051, DF480, evokes pressor responses with characteristics similar to those of noradrenaline, 179

—, DF480, γ-Piperidinobutyramide, evaluation of novel pressor activity of, 179

—, pressor responses of, not elicited by an α-adrenoceptor mechanism, 179

X

^{133}Xe-clearance, measurement of blood flow in rabbit skin homografts and autografts using a, technique, 396P

Xylometazoline, causes hyperpolarization and potentiation in guinea-pig liver slices, 362P

Y

Yohimbine, effect of, on adrenaline- and noradrenaline-induced effects on isolated rat vas deferens, 169

British Journal of Pharmacology

EDITED FOR

THE BRITISH PHARMACOLOGICAL SOCIETY

BY

A. W. Cuthbert *(Chairman)* H. M. Adam *(Secretary)*
Margaret Day *(Press Editor)*
J. D. Stephenson *(Assistant Press Editor)*

A. M. Barrett
W. G. Bisset
T. B. Bolton
P. B. Bradley
D. A. Brown
D. A. Callingham
T. J. Crow
F. de Matteis
C. T. Dollery
V. Eisen
E. W. Gill
A. F. Green
D. F. Hawkins
Margarethe Holzbauer
D. B. Hope
J. S. Kelly
G. M. Lees
G. P. Lewis
I. H. M. Main
E. K. Matthews
J. F. Mitchell
P. A. Nasmyth
M. W. Parkes
M. Sandler
A. P. Silverman
M. A. Simmonds
F. M. Sullivan
A. Ungar
R. A. Webster

VOLUME 56, 1976

LONDON
MACMILLAN JOURNALS LIMITED
4 LITTLE ESSEX STREET, WC2R 3LF

British Journal of Pharmacology

CONTENTS

VOLUME 56 NUMBER 1 JANUARY 1976

3 SCHILD, H.O. Dale and the development of pharmacology
9 HILL, R.G., SIMMONDS, M.A. & STRAUGHAN, D.W. Antagonism of γ-aminobutyric acid and glycine by convulsants in the cuneate nucleus of cat
21 GANGULY, D.K. Antioxotremorine action of propranolol
25 BOSIN, T.R., HIXSON, E. JANE & MAICKEL, R.P. Pressor effects of tryptamine analogues
29 OSBORNE, N.N. & PENTREATH, V.W. Effects of 5,7-dihydroxytryptamine on an identified 5-hydroxytryptamine-containing neurone in the central nervous system of the snail *Helix pomatia*
39 GRYGLEWSKI, R.J. & KORBUT, R. Bioassay of histamine in the presence of prostaglandins
43 ADAMS, M.D., CHAIT, L.D. & EARNHARDT, J.T. Tolerance to the cardiovascular effects of Δ^9-tetrahydrocannabinol in the rat
49 GILBERT, J.C. & WYLLIE, M.G. Effects of anticonvulsant and convulsant drugs on the ATPase activities of synaptosomes and their components
59 JOHNSON, A.M., LOEW, D.M. & VIGOURET, J.M. Stimulant properties of bromocriptine on central dopamine receptors in comparison to apomorphine, (+)-amphetamine and -DOPA
69 BUCKINGHAM, R.E., HAMILTON, T.C. & MOORE, R.A. Prolonged effects of *p*-chlorophenylalanine on the blood pressure of conscious normotensive and DOCA/saline hypertensive rats
77 MILSON, J.A. & PYCOCK, C.J. Effects of drugs acting on cerebral 5-hydroxytryptamine mechanisms on dopamine-dependent turning behaviour in mice
87 JOHNSON, F.N. The effect of lithium chloride on one-trial passive avoidance learning in rats
93 BARBER, H.E., CALVEY, T.N., MUIR, K.T. & TAYLOR, K. The effects of edrophonium on erythrocyte acetylcholinesterase and neuromuscular function in the rat
101 STONE, T.W. Responses of neurones in the cerebral cortex and caudate nucleus to amantadine, amphetamine and dopamine
111 FELTZ, ANNE & LARGE, W.A. Effect of atropine on the decay of miniature end-plate currents at the frog neuromuscular junction
115 DANILOV, A.F., IVANOV, YU.J. & MICHELSON, M.J. The influence of motor nerve tetanization on the effect of organophosphorus cholinesterase inhibitors on neuromuscular transmission

SHORT COMMUNICATIONS

125 BAKHLE, Y.S. & YOUDIM, M.B.H. Metabolism of phenylethylamine in rat isolated perfused lung: evidence for monoamine oxidase 'type B' in lung
128 BROWN, D.A. & KWIATKOWSKI, D. A note on the effect of dithiothreitol (DTT) on the depolarization of isolated sympathetic ganglia by carbachol and bromo-acetylcholine

VOLUME 56 NUMBER 2 FEBRUARY 1976

133 MARLEY, E. & WHELAN, JENNIFER E. Some pharmacological effects of *p*-chlorophenylalanine unrelated to tryptophan hydroxylase inhibition.
145 BUTTAR, H.S. Impairment in the hepatic clearance of [^{35}S]-bromosulphophthalein in paracetamol-intoxicated rats.

155 BRANDTZAEG, P., GAUTVIK, K.M., NUSTAD, K. & PIERCE, J.V. Rat submandibular gland kallikreins: purification and cellular localization.

169 JURKIEWICZ, A. & JURKIEWICZ, N.H. Dual effect of α-adrenoceptor antagonists in rat isolated vas deferens.

179 ALPS, B.J., DEVOY, P.W. & WATERFALL, J.F. The evaluation of the novel pressor activity of γ-piperidinobutyramide (Wy 20051, DF480).

187 SAXENA, P.N. Sodium and calcium ions in the control of temperature set-point in the pigeon.

193 EVANS, JANE P.M., GRAHAME-SMITH, D.G., GREEN, A.R. & TORDOFF, ANN F.C. Electroconvulsive shock increases the behavioural responses of rats to brain 5-hydroxytryptamine accumulation and central nervous system stimulant drugs.

201 SEIDLER, F.J. & SLOTKIN, T.A. Effects of chronic nicotine administration on the denervated rat adrenal medulla.

209 MICHAL, F. & MOTAMED, MINA. Shape change and aggregation of blood platelets: interaction between the effects of adenosine diphosphate, 5-hydroxytryptamine and adrenaline.

219 ABAITEY, A.K. & PARRATT, J.R. Cardiovascular effects of diethylcarbamazine citrate.

229 FLOWER, R.J., HARVEY, E.A. & KINGSTON, W.P. Inflammatory effects of prostaglandin D_2 in rat and human skin.

235 BUCKINGHAM, JULIA C. & HODGES, J.R. Hypothalamo-pituitary adrenocortical function in the rat after treatment with betamethasone.

VOLUME 56 NUMBER 3 MARCH 1976

45 KOSS, M.C. & NAKANO, J. Reflex bradycardia and hypotension produced by prostaglandin $F_{2\alpha}$ in the cat

255 ASHFORD, J. & JONES, B.J. The effects of intra-amygdaloid injections of 6-hydroxydopamine on avoidance responding in rats

63 LEES, P. & SERRANO, L. Effects of azaperone on cardiovascular and respiratory functions in the horse

271 CLARKE, D.E. ROMANYSHYN, W.M. Blood pressure responses to adrenal field stimulation as a measure of adrenal catecholamine release

279 DORRIS, R.L. & SHORE, P.A. On the mechanism of action of clozapine on the adrenergic neurone

285 GUTMAN, Y. & MAZUR-RUDER, M. Rat uterus renin-like activity: effect of stimuli and hormones

293 HIMORI, N. & TAIRA, N. A method for recording smooth muscle and vascular responses of the blood-perfused dog trachea *in situ*

301 RIVERA-CALIMLIM LEONOR. Impaired absorption of chlorpromazine in rats given trihexyphenidyl

307 CURTIS, D.R., GAME, C.J.A. & LODGE, D. Benzodiazepines and central glycine receptors

313 DICK, W.C., GRENNAN, D.M. & ZEITLIN, I.J. Studies on the relative effects of prostaglandins, bradykinin, 5-hydroxytryptamine and histamine on the synovial microcirculation in dogs

317 CHAHL, LORIS A. & LADD, R.J. The effects of prostaglandins E_1, E_2 and $F_{2\alpha}$ on the cutaneous vasculature of the rat

323 BOWERY, N.G. & JONES, G.P. A comparison of γ-aminobutyric acid and the semi-rigid analogues 4-aminotetrolic acid, 4-aminocrotonic acid and imidazole-4-acetic acid on the isolated superior cervical ganglion of the rat

331 ANDERSON, A.A. & LEES, G.M. Investigation of occurrence of tolerance to bronchodilator drugs in chronically pretreated guinea-pigs

339P PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY, London, 17–19 December, 1975

PROCEEDINGS OF THE BRITISH PHARMACOLOGICAL SOCIETY

Royal College of Surgeons, 17th–19th December, 1975

339P JONES, R.L. Selectivity of action of some C15-modified prostaglandins D

340P WHITTLE, B.J.R. Prostaglandins and changes in the gastric mucosal barrier and blood flow during indomethacin- and bile salt-induced mucosal damage

341P WILLIAMS, T.J. (introduced by Lewis, G.P.). The pro-inflammatory activity of E-, A-, D- and F-type prostaglandins and enalogues 16, 16-dimethyl-PGE$_2$ and (15S)-15-methyl-PGE$_2$ in rabbit skin; the relationship between potentiation of plasma exudation and local blood flow changes

342P CHANG, J., LEWIS, G.P. & PIPER, PRISCILLA J. The effects of anti-inflammatory steroids on levels of prostaglandin in adipose tissue *in vitro*

343P BLACKWELL, G.J. & FLOWER, R.J. Effects of steroid hormones on tissue levels of prostaglandin 15-hydroxydehydrogenase in the rat

244P BUNTING, S., MONCADA, S., NEEDLEMAN, P. & VANE, J.R. Formation of prostaglandin endoperoxides and rabbit aorta contracting substance (RCS) by coupling two enzyme systems

345P FORD-HUTCHINSON, A.W., SMITH, M.J.H. & WALKER, J.R. Chemotactic activity of solutions of prostaglandin E$_1$

346P BAKER, DOROTHEA J, TRIST, D.G. & WEATHERALL, M. Inhibition of phagocytosis by mepacrine

347P ASSEM, E.S.K. Inhibition by antigen and by histamine antagonists of the uptake of histamine by isolated human leucocytes

349P BAKHLE, Y.S. The nature of the bradykinin inactivating system in isolated lungs

350P MORTON, I.K.M., . ROSE, G.E., SAVERYMUTTU, S.H. & WOOD, J.R. Effects of some fatty acids on gallbladder function

351P AMBACHE, N. & KILLICK, S.W. Species variation in motor transmission to the retractor penis muscle

352P NISTRI, A. Effects of morphine on acetylcholine release from the frog spinal cord

353P GARTHWAITE, J. (introduced by Brown, D.A.). The uptake of weak acids and bases into isolated rat superior cervical ganglia in relation to intracellular pH

354P CHAPPLE, D.J., ENDERBY, D.H., HUGHES, R. & PAYNE, J.P. Neuromuscular and cardiovascular studies with dimethyl tubocurarine in anaesthetized cats, rhesus monkeys and man

355P HIROM, P.C., MILLBURN, P. & PARKER, R.J. (introduced by Williams, R.T.). The enterohepatic circulation of ^{1}H-phenolphthalein in the rat

356P RICHARDSON, B.P. (introduced by Berde, B.). The effects of cyproheptadine pretreatment on insulin release from isolated pancreatic islets

357P WAN, BEATRICE Y.C. (introduced by Black, J.W.). Effects of dibutyryl cyclic AMP and phosphodiesterase inhibitors on acid secretion by mouse stomach *in vitro*

358P RICHARDSON, P.D.I. The effects of int venous secretin on the small intestinal vasculatu of the cat

359P DUNCAN, A.J. & WILSON, W.S. Some pr servatives in eyedrop preparations hasten the formation of dryspots in the rabbit cornea

360P HOLMES, S.W. & SUGDEN, D. The effect o melatonin on pinealectomy-induced hypertensio in the rat

361P MELROSE, JOANNE, PALFREYMAN, M.G. POYSER, R.H. & WHITING, R.L. BRL 13776: a novel antihypertensive agent with interesting noradrenaline-depleting properties

362P BILSKI, A. & WALE, J. (introduced by Dawes, P.M.). Two types of intrinsic sympathomimetic activity with β-adrenoceptor blocking drugs

362P KOLLER, K. (introduced by Jenkinson, D.H.). An interaction between the α and β actions of catecholamines in guinea-pig liver slices

363P COLLIER, P.S. & SHANKS, R.G. Alterations t vasodilator effects of isoprenaline in the dog following intra-arterial infusions with isoprenaline, salbutamol and orciprenaline

364P BLAKELEY, A.G.H. & SUMMERS, R.J. The effects of AH 5158 on the overflow of transmitter and the uptake of [^{3}H]-(–)-noradrenaline in the cat spleen

365P DARGIE, H.J., FRANKLIN, S.S. & REID, J.L. The sympathetic nervous system and renovascular hypertension in the rat

366P FINCH, L. & HERSOM, A. Studies on the centrally mediated cardiovascular effects of apomorphine in the anaesthetized rat

366P BARASI, S., PARRY, O. & ROBERTS, M.H.T. The interpretation of responses of motoneurone field potentials to 5-hydroxytryptamine

367P GOODLET, I., MIREYLEES, S.E. & SUGRUE, M.F. The selective inhibition of 5-hydroxytryptamine re-uptake by Org 6582

368P CURZON, G. & MARSDEN, C.A. Effect of LSD on rat brain 5-hydroxytryptamine metabolism at elevated environmental temperature

370P LITTLETON, J.M. & MACLEAN, K.I. (introduced by Brownlee, G.). Alterations in dopamine uptake in rat corpus striatum induced by combinations of stress and Δ^8-tetrahydrocannabinol (Δ^8-THC)

71P JATON, A.L., LOEW, D.M. & VIGOURET, J.M. CF 25-397 (9, 10-Didehydro-6-methyl-8β-[2-pyridylthiomethyl]ergoline), a new central dopamine receptor antagonist

372P FRY, J.P., SHARMAN, D.F. & STEPHENS, D.B. The ontogenesis of cerebral dopamine metabolism in the pig

373P DONALDSON, I.M., JENNER, P., MARSDEN, C.D., MILLER, R. & PERINGER, E. Is metoclopramide a directly acting dopamine receptor antagonist?

374P CANDY, J.M. & KEY, B.J. Blockade of the electrocortical changes induced by perfusion of amphetamine in the brain stem reticular formation

375P YOUDIM, M.B.H. Monoamine oxidase active site; the binding to and titration of monoamine oxidase with [^{14}C]-selective inhibitors

375P HILLIDGE, C.J. & LEES, P. Influence of etorphine acepromazine and diprenorphine on cardiovascular function in ponies

376P GRANT, L. & POWIS, G. The effect of inhibitors of alcohol metabolism on the changes in the hepatic microsomal metabolism of foreign compounds produced by a single dose of ethanol in the rat

377P ABU-MURAD, C., GRIFFITHS, P.J. & LITTLETON, J.M. (introduced by Brownlee, G.). Catecholamine metabolism and liver dysfunction during induction of ethanol dependence

378P FILE, SANDRA E. (introduced by Neal, M.J.). Are central cholinergic pathways involved in the habituation of exploration and distraction?

379P AMADUCCI, L., MANTOVANI, P. & PEPEU, G. Effect of some phospholipids on acetylcholine output from the cerebral cortex in the rat

380P NAHORSKI, S.R., PATTON, WENDY & ROGERS, K.J. Developmental changes in the sensitivity of neurohormone-stimulated cyclic AMP formation in chick cerebral hemispheres

381P ANLEZARK, G.M., MARSDEN, C.D. & MELDRUM, B.S. A primate model of acute dystonic reaction to neuroleptics

382P COLLIER, H.O.J. & FRANCIS, D.L. Stereospecific suppression by opiates of the quasi-morphine abstinence syndrome elicited by 3-isobutyl-l-methylxanthine (IBMX)

382P HOLZBAUER, MARGARETHE. Physiological aspects of the hypnotic properties of steroid hormones.

383P ANLEZARK, G.M., HORTON, R.W., MELDRUM, B.S., SAWAYA, M.C.B. & STEPHENSON, J.D. γ-Aminobutyric acid metabolism and the anticonvulsant action of ethanolamine-o-sulphate and di-n-propylacetate

385P LEWIS, A.J. The assessment of systemically and topically administered anti-inflammatory drugs using u.v. erythema production in the rat

386P CUTHBERT, N.J., DINNEEN, L.C., FRANCIS, D.L. & SCHNEIDER, C. A rapid *in vivo* test for dependence potential of analgesic drugs

386P HAEUSLER, G. & LEWIS, P.J. Sympathetic nerve recording in the conscious rabbit

387P BRADLEY, N.J. & POULTER, L.W. (introduced by Lewis, G.P.). *In vitro* methods of assessing the physiological activation of macrophages *in vivo*

388P FRY, J.P., SHARMAN, D.F. & STEPHENS, D.B. The effect of apomorphine on oral behaviour in piglets

388P FARRINGTON, HELEN E. & OWEN, D.A.A. Measurement of vascular changes in acute inflammatory responses

389P BUSHMAN, J.A., ENDERBY, D.H., FOWLER, K.T. & PAYNE, J.P. The use of a mass spectrometer for the analysis and measurement of anaesthetic vapours

390P ENDERBY, D.H., HUGHES, R., PAYNE, J.P. & WORSLEY, R. Assessment of neuromuscular blockade in man by use of tetanic and single twitch contractions of the adductor pollicis muscle

391P HUTCHINGS, R.T., LEWIS, G.P., SABIKOWSKI, Z.T. & WESTWICK, J. Measurement of changes in the microvasculature

391P NIEDERHAUSER, U. & WHITTLE, B.J.R. A simple perfusion system for the study of histamine release from rat peritoneal mast cells

392P ADEBANJO, A.O., AMBACHE, N. & VERNEY, J. The inhibitory transmission to the internal anal sphincter

394P EDWARDS, J.H., EVANS ELIZABETH, EVANS, P.H., NICHOLLS, P.J. & RAJAN, K.T. Effect of a purified cotton dust extract on human lung in culture

395P DOLPHIN, A.C., ELLIOTT, P.N.C. & JENNER, P. Reduction of locomotor activity in mice by dopamine-β-hydroxylase inhibitors — evidence against the involvement of non-specific irritation

395P McGRATH, J.C. & MacKENZIE, J.E. A pithed rabbit preparation for stimulation of different segments of the autonomic outflow

396P BAKER, J.B.E. The episcope for large screen projection of tracings on opaque media during their recording for demonstrations

396P PECK, M.J. & WILLIAMS, T.J. (introduced by Lewis, G.P.). Measurement of inflammatory exudation and local blood flow changes in rabbit skin, using [^{131}I]-albumin and ^{133}Xe

396P LEWIS, G.P., PECK, M.J., WILLIAMS, T.J. & YOUNG, BEVERLEY A. Measurement of blood flow in rabbit skin homografts and autografts using a ^{133}Xe-clearance technique

396P McCALL ELAINE & YOULTEN, L.J. Prostaglandin production by rat polymorphonuclear leucocytes

VOLUME 56 NUMBER 4 APRIL 1976

399 SMITH, D.F. Lithium orotate, carbonate and chloride: pharmacokinetics, polydipsia and polyuria in rats.

403 KAROUM, F. MAJCHROWICZ, E. & WYATT, R. Brain concentrations of biogenic amine metabolites in acutely treated and ethanol-dependent rats.

413 EGAN, SANDRA M., GRAHAM, J.D.P. & LEWIS, M.J. The uptake of tritiated Δ^1-tetrahydrocannabinol by the isolated vas deferens of the rat.

417 DRUMMOND, A.H. & GORDON, J.L. Uptake of 5-hydroxytryptamine by rat blood platelets and its inhibition by adenosine 5′-diphosphate.

423 MARCHAND, C. & NADEAU, D. The distribution and kinetics of sulphacetamide in leukaemic mice.

431 CARRUBA, M.O., GROPPETTI, A., MANTEGAZZA, P., VICENTINI, L. & ZAMBOTTI, F. Effects of mazindol, a non-phenylethylamine anorexigenic agent, on biogenic amine levels and turnover rate.

437 DUTTA, S., MARKS, B.H. & STEPHEN, P.M. The uptake and subcellular distribution of radiolabelled metabolites of digitoxin in the guinea-pig isolated perfused heart.

443 DOW, R.C. & LASZLO, I. Uptake of 5-hydroxytryptamine in different parts of the brain of the rabbit after intraventricular injection.

449 CARRIER, O., JR., & HESTER, R.K. The relationship between calcium and increased sensitivity of rabbit aortae four hours after reserpine.

457 MICHELSON, M.J. & SHELKOVNIKOV, S.A. Isotonic and isometric responses of different tonic muscles to agonists and antagonists.

469 FERREIRA, S.H., HERMAN, A.G. & VANE, J.R. Prostaglandin production by rabbit isolated jejunum and its relationship to the inherent tone of the preparation.

479 MAGOS, L. The effects of dimercaptosuccinic acid on the excretion and distribution of mercury in rats and mice treated with mercuric chloride and methylmercury chloride.

485 GRAY, J.A., LÜLLMANN, H., MITCHELSON, F. & REIL, G.-H. Stereoselective binding in cardiac tissue of the enantiomers of benzetimide, an antimuscarinic drug.

SHORT COMMUNICATIONS

491 KUMAR, A., SHARMA, H.L. & SHARMA, V.N. Effect of SAS (A new 10-*N*-acylaminophenothiazine) on gastric secretion and ulceration in rats.

494 CHIPMAN, P. & GLOVER, W.E. Histamine H_2-receptors in the human peripheral circulation.

INSTRUCTIONS TO AUTHORS

Papers will be considered for publication on all aspects of drug action, including chemotherapy. A recent issue of the Journal is a good guide to style.

Manuscripts (two copies) should be sent to Dr H.M. Adam, Department of Pharmacology, University of Edinburgh Medical School, 1 George Square, Edinburgh, EH8 9JZ, from whom copies of the full Instructions to Authors, published in *Br. J. Pharmac,* **50**, 3-23, may also be obtained. Specific points to note are:

1. **Manuscript** should be typed in double spacing on one side of paper not larger than A4 (206 x 294 mm). There should be a separate title page giving the names and addresses of the authors **in alphabetical order.** A short title of not more than 50 letters should also be suggested.

2. **Statement** Papers are accepted only if accompanied by a statement that they have not been and will not be published in whole or in part in any other Journal.

3. **Summary** (2 copies) This should be a short summary of results and conclusions arranged in numbered paragraphs.

4. **Abstract** (2 copies) An abstract of about 100 words suitable for inclusion in an abstracting journal should be typed on a separate sheet, giving the author's name and address and the title of the paper.

5. **Figures** These should not be larger than A4 (206 x 294 mm) and may be in the form of original drawings, recorded tracings or high quality photographic prints made from them. Negative prints of kymograph tracings (black on white) should be provided, and both ordinates and abscissae should be calibrated. Lettering on figures should be inserted in pencil. Symbols which are to appear in figures or legends should be chosen from the following:

○ ● □ ■ △ ▲ ▽ ▼

Photographs and photomicrographs should be printed on glossy paper and should be larger than the size required for reproduction.

6. **Tables** These should not have more than 85 characters to a line (counting spaces between columns as 4 characters) and certainly not more than 110 characters to a line, unless absolutely unavoidable. **Figure legends** and **tables** should be typed on separate sheets of paper.

7. **References** These should be collected in alphabetical order at the end of the paper. They should include the authors' names, year of publication (in brackets), title of article, title of publication (abbreviated in accordance with the fourth edition of the *World List of Scientific Periodicals*), volume number and first and last page numbers. References to books should in addition, include the names of editors, the edition number, where appropriate, and the town of origin and name of publisher.

Two page proofs will be supplied, one of which may be retained by the authors. The other should be corrected immediately and returned to the Press Editor. Corrections should be kept to a minimum.

Twenty-five reprints will be supplied to the authors free of charge. Additional reprints can be purchased. A reprint order form, which should be returned promptly, will be sent out with the proofs. No reprints of abstracts are supplied.

Short Communications These may be accepted if they merit priority publication and do not require revision. They may contain up to 1,200 words of text and only one figure or table. The summary should be a single paragraph. An abstract and statement should be included.

British Journal of Pharmacology

VOLUME 56 NUMBER 4 APRIL 1976

399 SMITH, D.F. Lithium orotate, carbonate and chloride: pharmacokinetics, polydipsia and polyuria in rats.

403 KAROUM, F. MAJCHROWICZ, E. & WYATT, R.J. Brain concentrations of biogenic amine metabolites in acutely treated and ethanol-dependent rats.

413 EGAN, SANDRA M., GRAHAM, J.D.P. & LEWIS, M.J. The uptake of tritiated Δ^1-tetrahydrocannabinol by the isolated vas deferens of the rat.

417 DRUMMOND, A.H. & GORDON, J.L. Uptake of 5-hydroxytryptamine by rat blood platelets and its inhibition by adenosine 5′-diphosphate.

423 MARCHAND, C. & NADEAU, D. The distribution and kinetics of sulphacetamide in leukaemic mice.

431 CARRUBA, M.O., GROPPETTI, A., MANTEGAZZA, P., VICENTINI, L. & ZAMBOTTI, F. Effects of mazindol, a non-phenylethylamine anorexigenic agent, on biogenic amine levels and turnover rate.

437 DUTTA, S., MARKS, B.H. & STEPHEN, P.M. The uptake and subcellular distribution of radiolabelled metabolites of digitoxin in the guinea-pig isolated perfused heart.

443 DOW, R.C. & LASZLO, I. Uptake of 5-hydro tryptamine in different parts of the brain of rabbit after intraventricular injection.

449 CARRIER, O., JR., & HESTER, R.K. T relationship between calcium and increa sensitivity of rabbit aortae four hours after reserp

457 MICHELSON, M.J. & SHELKOVNIKOV, S Isotonic and isometric responses of different to muscles to agonists and antagonists.

469 FERREIRA, S.H., HERMAN, A.G. & VAN J.R. Prostaglandin production by rabbit isolat jejunum and its relationship to the inherent tone the preparation.

479 MAGOS, L. The effects of dimercaptosuccinic on the excretion and distribution of mercury in r and mice treated with mercuric chloride and met mercury chloride.

485 GRAY, J.A., LÜLLMANN, H., MITCHELS F. & REIL, G.-H. Stereoselective binding in ca tissue of the enantiomers of benzetimide, an muscarinic drug.

SHORT COMMUNICATIONS

491 KUMAR, A., SHARMA, H.L. & SHARMA, V Effect of SAS (A new 10-*N*-acylaminoph thiazine) on gastric secretion and ulceration in

494 CHIPMAN, P. & GLOVER, W.E. Histamine receptors in the human peripheral circulation.

Printed in Great Britain by The Whitefriars Press Ltd, London and Tonbridge